The Impact of Developmental Stress on The Functioning and Vulnerability of CNS neurons

By

Ilse-Sanet Pienaar

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The Faculty of Health Sciences

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Supervisor: Professor W.M.U. Daniels

Department of Medical Physiology, Stellenbosch University

Co-supervisor: Professor D.J. Stein

Department of Psychiatry, University of Cape Town

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DECLARATION

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OVERALL ABSTRACT

The overall objective of this thesis is to provide additional data to assist clinicians and experimental neurologists alike in the quest for better understanding, more accurately diagnosing and more successfully treating patients suffering from Parkinson's disease (PD). The general theme of the thesis is the interaction between certain environmental stimuli, including the exposure to adverse events during early central nervous system (CNS) development and the manifestation of elements of neurodegeneration, whether by means of neurochemical changes or expressed as a dysfunctional voluntary motor system.

The first chapter provides a general introduction to the research theme of the thesis. This includes, in particular, a discussion on current understanding concerning the etiology and clinical profile of PD, the relative contribution made by genetic factors compared to environmental ones, and current treatment strategies for treating the disease. Mention is also made of the failure of these therapeutic applications for reversing or protecting against the disease, due to the side-effects associated with them. The material covered in chapter 1 provides the basis for the more complete discussion concerning these various aspects, contained in the chapters to follow.

The overall aim was also to characterise the effects of commonly used toxin-induced animal models of PD, and the extent of vulnerability that the CNS displays towards them. The destruction of dopaminergic neurons following the administration of 6-OHDA at targeted points along the nigrostriatal tract is used extensively to model PD pathology in rats and is an established animal model of the disease. However, mature or even aged animals are mainly used in these studies, while the effects that the toxin might have on the developing CNS remain unclear. The study reported in chapter 4 aimed to elucidate some of 6-OHDA's actions on the young adolescent (35 days-old) CNS by comparing the motor and biochemical effects of a unilateral infusion of the toxin into two anatomically distinct basal ganglia loci: The medial forebrain bundle (MFB) and the striatum. Animals were randomly assigned to receive either a direct delivery of 6-OHDA (12µg/4µl) into the MFB or an indirect injection, into the striatum. Although both lesion types were used, the MFB model is considered a more accurate portrayal of end-stage PD, while the striatum-model better reflects the long-term progressive pathology of the disease. The different lesions' effects on motor function were determined by observing animal's asymmetrical forelimb use to correct for weigh shifting during the vertical exploration of a cylindrical enclosure. Following the final behavioral assessment, the concentration of dopamine (DA) and DA metabolites remaining in the post-mortem brains were determined using

HPLC electrochemistry (HPLC-EC) and the levels compared between the two groups. The HPLC-EC results revealed a compensatory effect for DA production and DA turnover on the lesioned hemisphere side of the toxin-infused animal group. Thus, following 6-OHDA treatment, there appears to be extensive adaptive mechanisms in place within the remaining dopaminergic terminals that may be sufficient for maintaining relatively high extracellular and synaptic concentrations of DA. However, since substantial changes in motor-function were observed, it is suggested that the capacity of the remaining dopaminergic neurons to respond to increased functional demands may be limited. In addition, the behavioral results indicate that the distinct indices relating to different functional deficits depend on the lesioning of anatomically distinct structures along the nigrostrial tract.

It has long been known that far fewer women are diagnosed with PD than men are. This seeming protection offered to females against degenerative disease of the CNS may relate to estrogen, although the hormone's mechanism of action on the dopaminergic system is poorly defined. With an estimated 10-15 million women using oral contraceptives (OCs) in the United States alone, the aim of chapter 2 was to examine the evidence for a possible relationship between PD and the female reproductive hormone estrogen. A review of the current literature available on the topic was performed by consulting Medline, and by performing a search of the case-reports contained within the World Health Organization's (WHO) International Drug Monitoring database, for possible PD-related symptoms that may arise from estrogen replacement therapy (ERT). The results, whilst conflicting, seem to suggest that estrogen protects women from obtaining the disease, or at least some features of it. Intensive research efforts are called for, with sufficient power to establish the relationship between ERT and the onset and development of parkinsonism. Chapter 3 reports on the results obtained from an experiment that subjected young Sprague-Dawley rats, 35 days of age, to a lower and a higher dose of 6-OHDA delivered to the MFB. Control rats received equivalent saline infusions. At 14 days post-surgery, the rats were evaluated for forelimb akinesia. For the higher dose of 6-OHDA the female rats were less impaired than males in making adjustment steps in response to a weight shift and in the vibrissae-evoked forelimb placing test. In addition, Tyrosine hydroxylase (TH) immunoreactivity was significantly higher for the female rats. Early gender differences in cell survival factors and/or other promoters of neuroplasticity may have contributed to the beneficial outcome seen in the females. For example, nerve growth factor (NGF) was found to be higher in the female rats following administration of the DA neurotoxin. It is unclear whether gonadal steroids are involved, and, if so, whether female hormones are protective or whether male hormones are prodegenerative. Determining the mechanisms for the improved outcome seen in the young female rats may lead to potential treatment strategies against PD.

Many studies have shown that early life stress may lead to impaired brain development, and may be a risk factor for developing psychiatric diseases, including clinical depression. However, few studies have investigated the impact that early stress may have on the onset and development of neurodegenerative disorders such as PD. The study reported on in chapter 5 conjointly subjected rat pups to a maternal separation (MS) paradigm that is a well characterised model of adverse early life events, and a unilateral, intrastriatal injection of 6-OHDA. The combined effects of these models on motor deficits and brain protein levels were investigated. Specifically, the animals were assessed for behavioral changes at 28 days post-lesion with a battery of tests that are sensitive to the degree of DA loss sustained. The results show that animals that had been subjected to MS display poorer performance in the vibrissae and single-limb akinesia test compared to non-MS control animals (that had also been subjected to the toxin exposure). In addition, there was a significant increase in the loss of TH staining in MS rats compared to non-MS ones. The results from this study therefore suggest that exposure to adverse experiences during the early stages of life may contribute towards making dopaminergic neurons more susceptible to subsequent insults to the CNS occurring during mature stages of life. Therefore, taken together, early exposure to stress may predispose an individual towards the onset and development of neurodegenerative disease, which especially becomes a threat during the later stages of adult life.

Moreover, within the framework of these characteristics, the capacity of a widely-used pharmacological agent (statins) was tested for possible future therapeutic application in PD (chapter 7). Although the precise cause of sporadic PD remains an enigma, evidence suggests that it may associate with defective activity of complex I of the mitochondrial electron transport chain. Mitochondrial DNA transmit and express this defect in host cells, resulting in increased oxygen free radical production, depressed antioxidant enzyme activities, and greater susceptibility to apoptotic cell death. Simvastatin is a member of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) group of drugs that are widely used for lowering cholesterol levels in patients who display elevated concentrations of low-density lipoprotein cholesterol. The study aimed to investigate the effects that statin-treatment have on motor-function and at the mitochondrial-protein level, using rotenone, a mitochondrial complex I inhibitor, as a rat-model of PD. Adult male Sprague-Dawley rats were treated either with simvastatin (6mg/day for 14 days) or with a placebo. Two different tests to assess motor function were used: The apomorphine-rotation test, and the vibrissae-elicited forelimb placement test. Following the drug administration protocol, the nigrostriatal tract was unilaterally lesioned with either rotenone (3 µg/4 µl) or, for the controls, were sham-operated by infusing the vehicle (DMSO:PEG) only. Five days later the rats were killed and a highly purified concentration of isolated mitochondria was prepared from the substantia nigra (SN) sections. 2-

Dimensional electrophoresis (2-DE) with subsequent identification of the spots using electronspray ionization quadruple time-of-flight mass spectrometrical (ESI-Q-TOF MS) was performed and the results BLAST-searched using bio-informatics tools for naming the identified peptides. The motor test results indicate that while unilateral rotenone causes behavioral asymmetries, treatment with simvastatin improved motor function relative to the rotenone-induced ones. Mass Spectroscopy identified 23 mitochondrial proteins that differ significantly in protein expression ($p < 0.05$) following simvastatin treatment. The altered proteins were broadly classified according to their cellular function into 6 categories, with the majority involved in energy metabolism. This study effectively illustrated how neuroproteomics, with its sophisticated techniques and non-biased ability to quantify proteins, provides a methodology with which to study the changes in neurons associated with neurodegeneration. As an emerging tool for establishing disease-associated protein profiles, it also generates a greater understanding as to how these proteins interact and undergo post-translational modifications. Furthermore, due to the advances made in bioInformatics, insight is created concerning their functional characteristics. Chapter 4 summarises the most prominent proteomics techniques and discuss major advances made in the fast-growing field of neuroproteomics in PD. Ultimately, it is hoped that the application of this technology will lead towards a presymptomatic diagnosis of PD, and the identification of risk factors and new therapeutic targets at which pharmacological intervention can be aimed.

The final chapter (chapter 8) provides a retrospective look at the academic work that had been performed for the purpose of this thesis, recaps on the main findings, and also highlights certain aspects of the project and provides relevant suggestions for future research. Lastly, the appendix provides a detailed overview of the methods followed for the experiments described in this thesis. It provides not only a comprehensive description of the techniques that had been followed, but provides information concerning the care taken with the animals (i.e. post-surgery) in order to control for the potential influence of experimental variables on the results.

ALGEHELE OPSOMMING

Die algemene doel van die tesis is om addisionele data te verskaf ten einde klinisie en eksperimentele neuroloë van hulp te verskaf ten einde Parkinson se siekte (PS) beter te verstaan, meer akkuraat te diagnoseer en meer suksesvol te behandel. Die algemene tema van die tesis is die interaksie tussen bepaalde omgewings stimuli, insluitende blootstelling aan adverse faktore tydens die sentrale senuwee stelsel (SSS) se ontwikkeling en die manifestasie van elemente van neurodegenerasie, hetsy d.m.v. neurochemiese veranderings of uitgedruk as ‘n disfunksionele willekeurige motor stelsel.

Hoofstuk 1 verstaf ‘n algemene inleiding tot die navorsings-tema van die tesis. Dit sluit in spesifieke bespreking van die huidige verstandhouding rakende die etiologie en kliniese profiel van PS, die relatiewe bydra gemaak deur genetiese faktore vergeleke met omgewings faktore, asook die huidige behandelings-strategiëe waarmee die siekte behandel word. Melding word ook gemaak van die gebrek aan sukses met die bepaalde terapeutiese applikasies ten einde teen die siekte te beskerm of sy progressie te vertraag. Die material gedek in hoofstuk 1 verskaf die basis vir die meer omslagtige bespreking rakende die verskillende aspekte, wat volg in die daaropvolgende hoofstukke.

Die algemene doel was ook om die effekte van algemeen gebruikte toksien-geinduseerde diere modelle van PS asook die mate van weerloosheid wat die SSS daarteenoor toon te karakteriseer. Die vernietiging van dopaminergiese neurone deur die administrasie van 6-OHDA by geteikende punte in die nigrostriatale neuronale baan, word ekstensief gebruik ten einde PS te modeler in rotte en verteenwordig ‘n gevestigde diere model van die siekte. Volwasse of verouderde diere word egter meestal gebruik in die studies, terwyl die effekte wat die toksien het op die ontwikkelende SSS steeds onduidelik is. Die studie gerapporteer in hoofstuk 4 se doel was om sommige van die effekte van ‘n unilaterale infusie van 6-OHDA op jong adolessente diere (35 dae oud) in 2 anatomiese aparte basale ganglia loki: Die mediale voorbrein bondel en die striatum, te omskryf i.t.v. die motoriese en biochemiese effekte. Diere is verdeel in 2 groepe, wat of ‘n dosis 6-OHDA (12µg/4µ) direk togedien is in die mediale voorbrein bondel, of indirek, deur intespuit in die striatum. Alhowel albei letsel modelle gebruik word, word die mediale voorbrein bondel model beskou as ‘n meer akkurate voorstelling van eind-fase PS, terwyl die striatum-model die lang-termyn progressiewe patologie van die siekte beter reflekteer. Die verskillende effekte van die letsels op motor gedrag is bepaal deur die diere se asimmetriese voor-ledemaat gebruik tydens die vertikale eksplorasie van ‘n silinder te analiseer. Na die finale gedrags ontleding, is die konsentrasie dopamien, en dopamien-metaboliete bepaal in die post-mortem breine d.m.v. HPLC-elektrochemie (HPLC-EC) en die vlakke is vergelyk tussen die 2 groepe. Die HPLC-EC resultate het ‘n kompenserende effek vir

dopamien-produksie en -metabolisme getoon aan die geletselde kant van die toksien geadministreerde diere. Dus, na die 6-OHDA behandeling, blyk dit of daar ekstensiewe aanpassings meganismes te werke is binne die oorblywende dopaminergiese terminale wat moontlik voldoende is ten einde relatiewe hoë ekstrasellulere en sinaptiese konsentrasies van dopamien te handhaaf. Dit blyk egter, aangesien groot verskille in motoriese funksie geobserveer is, dat die oorblywende neurone se vermoë om te reageer op stygende funksionele eise beperk is. Hiermee saam dui die gedrags resultate aan dat aparte meetings-indekse, blykbaar verwant aan die verskillende funksionele tekorte, afhanklik is van watter anatomies-aparte struktuur, inherent tot die nigrostriatale neuronale baan, geletsel is.

Dit is reeds lank bekend dat heelwat minder vrouens gediagnoseer word met PS as mans. Hierdie blykbare beskerming teen degeneratiewe siektes van die SSS mag verband hou met estrogeen, alhowel die hormoon se meganisme van effek op die dopaminergiese stelsel onvolledig gedefinieerd is. Met ‘n geskatte 10-15 miljoen vrouens wat van orale kontrasepsie middels gebruik maak in die VSA alleen, was die doel van hoofstuk 2 van die tesis om ondersoek intestel na die bewyse vir ‘n moontlike verband tussen PS en die vroulike gonodale hormoon estrogeen. ‘n Soektog na die huidig-beskikbare literatuur rakende die onderwerp is uitgevoer. Die Medline databasis is gekonsulteer en ‘n soektog is ook uitgevoer op die gevalle-studies ingesluit in die Wêreld Gesondheids Organisasie se Internasionale Medisyne-Middel Moniterings Databasis, vir moontlike PS-verwante simptome wat mag voorkom a.g.v. estrogeen vervangings terapie (EVT). Die resultate, alhoewel konflikterend, dui daarop dat estrogeen vrouens beskerm teen die siekte, of minstens sekere aspekte daarvan. Intensiewe navorsings pogings is nodig met genoeg statistiese krag om die verband te ontleed tussen EVT en die ontstaan en ontwikkeling van PS. Hoofstuk 3 rapporteer die resultate van ‘n eksperiment wat jong Sprague-Dawley rotte, 35 dae oud, onderwerp het aan ‘n laer vs. ‘n hoër dosis 6-OHDA gelewer aan die mediale voorbrein bondel. Kontrole rotte het ‘n gelywaardige hoeveelheid van die toksiese middel se vervoerstof (“saline”) ontvang. Teen 14 dae na die sjirurgie is die rotte geëvalueer vir voor-ledemaat akinesie. Vir die hoër dosis 6-OHDA was die vroulike rotte minder geinhibeer as die manlike diere om aanpassings-stappe te maak in respons tot ‘n gewigsverskuiwing asook in die “vibrissae-evoked forelimb placing test”. Tirosien hidroksielase (TH) immunoreaktiwiteit was statisties hoër as vir die vroulike rotte. Geslags verskille in sel oorlewing en ander promoters van neuroplastisiteit, met moontlike oorsprong uit die neonatale of selfs pre-neonatale tydperk mag bygedra het tot die voordelige effek toegestaan aan die vroulike rotte. Byvoorbeeld, ‘n opgradering van neuronale groei factor (NGF) was gevind in die vroulike rotte na administrasie van die dopaminergiese neurotoksien. Dit is onduidelik of gonodale steroïd hormone betrokke is by die effek, en indien wel, of vroulike hormone beskerming verskaf en of manlike hormone pro-degeneratief is. Deur die meganismes betrokke

in die finale uitkoms van die jong vroulike rotte te bepaal, mag lei tot potensiele behandelings strategiëe vir PS.

Verskeie studies het getoon dat vroëe lewens stres lei tot ‘n nadelige effek op brein ontwikkeling en mag ‘n risiko faktor wees in die ontwikkeling van verskeie psigatriese siektes, ondermeer kliniese depressie. ‘n Beperkte aantal studies het egter die impak van vroëe lewens-stress op die ontstaan en ontwikkeling van neurodegeneratiewe siektes soos PS bestudeer. Die studie gerapporteer in hoofstuk 5 het gesamentlik rot neonate onderwerp aan ‘n maternale separasie (MS) paradigma, wat ‘n goed gekaraktiseerde model is van adverse vroëe lewens gebeure, asook ‘n unilaterale, intra-striatale inspuiting van 6-OHDA. Die effekte van die gekombineerde modelle op motoriese gedrag en brein protein vlakke is ondersoek. Die diere is spesifiek geëvalueer vir gedrags veanderinge teen 28 dae na afloop van die sjirurgie met ‘n battery toetse wat sensitief is vir die mate van dopamien verlore. Die resultate het gewys dat die rotte onderwerp aan MS swakker presteer het in die toetse in vergelyking met die kontrole rotte wat nie blootgestel is aan MS nie, maar wel die 6-OHDA intra-serebrale inspuiting ontvang het. Daarmee saam was daar ‘n statisties beduidende toename in die verlies van TH in die MS rotte. Die resultate van die studie dui dus daarop dat blootstelling aan adverse lewensgebeure tydens die vroëe lewens-jare mag bydra daartoe om dopamienergiese neurone meer weerloos te maak teenoor daaropvolgende effekte wat spruit uit blootstelling aan adverse ervarings tydens die volwasse lewensjare. Saamgevat dui die resultate van die studie dus daarop dat blootstelling aan stress mag die individu predispositioneer tot die onstaan en ontwikkeling van neurodegeneratiewe siektes, wat toeneem in risiko tydens die latere lewensfases.

Daarmee saam, binne die raamwerk van die karakter-eienskappe kenmerkend van die diere-modelle van PS, is daar ondersoek ingestel na die vermoë van ‘n farmakologiese middel (statiene) wat wye gebruik vind as ‘n potensieel toekomstige terapeutiese middel in PS (hoofstuk 7). Alhowel die definitiewe rol van PS enigmaties bly, dui verskeie studies daarop dat dit moontlik assosieer met ‘n defektiewe aktiwiteit van kompleks I van die mitokondriale elektron vervoer ketting. Die defek word deur mitokondriale DNS uitgedruk in selle, wat lei tot ‘n toename in suurstof vry radikale produksie, onderdrukte anti-oksidant ensiem aktiwiteite, asook groter weerloosheid teenoor apoptotiese sel-dood. Simvastatin is ‘n lid van die 3-hydroxy-3-methylglutaryl koënsiem A (HMG-CoA) reduktase inhibeerders (statiene) groep middels wat gebruik word om cholesterol vlakke te verlaag in pasiënte wat lei aan patologiese hoë vlakke van lae-digtheid lipoprotein cholesterol. Die studie het ten doel gehad om die effekte wat statiene het op motoriese gedrag en op die mitokondriale protein-vlak te ondersoek, deur rotenoon, ‘n mitochondriale kompleks I inhibeerder te gebruik as a rot model van PS. Volwasse manlike Sprague-Dawley rotte is behandel hetsy met simvastatin (6mg/dag vir 14 dae) of met ‘n placebo. Twee verskillende toetse ten einde motor funksies te evalueer is gebruik: Die

apomorfien-rotasie toets en die “vibrissae-elicited forelimb placing test”. Na die statien administrasie van 14 dae is die nigrostriatale neuronale baan unilateraal met hetsy rotenoon (3 µg/4 µl) of, vir die kontrole diere, slegs met die vervoerstof van die toksien (DMSO:PEG) geletsel. Vyf dae later is die rotte gedekapiteer en ‘n hoë konsentrasie van geisoleerde mitokondria is voorberei vanaf die substantia nigra seksies. 2-Dimensionele elektroforesis met “eletronspray ionisation” kwadrupool tyd-van-vlug massa spektrometriese (ESI-Q-TOF-MS) identifikasie van die “spots” was uitegevoer en die resultate is geBLAST soek m.b.v. bio-informatieka sagteware, ten einde die geidentifiseerde peptiede te benoem. Die motoriese toets-resultate dui daarop dat alhoewel die unilaterale infusie van rotenoon gedrags-asimmetrie veroorsaak, behandeling met simvastatin dié gedrag verbeter in vergelyking met die placebo en rotenoon behandelde diere. Massa spektroskopie het altesaam 23 proteine geïdentifiseer wat statisties belangrik verskil i.t.v. protein ekspressie ($p < 0.05$), as ‘n resultaat van simvastatin behandeling. Die proteine wat verandering getoon het is breedweg geklassifiseer in 6 katergorïee volgens hul sellulêre funksie, met die meerderheid wat betrokkenheid toon by energie metabolisme. Hierdie studie het effektief aangetoon hoe “neuroproteomics”, met sy gesofistikeerde tegnieke en onsydige vermoë om proteine te kwantifiseer, ‘n metodologie verskaf waarmee die veranderinge in neurone wat assosieer met neurodegenerasie, bestudeer kan word. As ‘n ontwikkelende tegniek vir die bevestiging van siekte-geassosieerde protein profiele, genereer dit ook groter insig rakende hoe die proteine inter-reageer en post-translasie veranderings ondergaan. Hiermee saam het die verbeteringe gebring in die area van bio-informatika insig geskep rakende proteine se funksionele eienskappe. Hoofstuk 4 som die mees prominente proteomiese tegnieke op en bespreek die mees belangrike verbeteringe in die snel-groeiende veld van “neuroproteomics” in PS. Die uiteindelike hoop is dat die tegnologie aangewend kan word ten einde ‘n presimptomatiese diagnose van PS met meer sekerheid te kan maak, asook die identifikasie van risiko faktore en nuwe terapeutiese teikens waarop farmakologiese intervensie gerig kan word.

Die finale hoofstuk (hoofstuk 8) verskaf ‘n retrospektiewe blik op die akademiese werk uitegevoer vir die tesis, som die hoof- bevindinge op, terwyl dit ook sekere aspekte van die projek benadruk en relevante voorstelle maak vir toekomstige navorsing. Laastens verskaf die appendiks ‘n gedetailleerde oorsig van die metodes gevolg vir die eksperimente wat beskryf word in die tesis. Hoofstuk 8 verskaf nie net ‘n komprehensiewe beskrywing van die tegnieke gevolg nie, maar verskaf ook informasie rakende die sorg verskaf aan die diere (bv. na afloop van die sjirurgie) ten einde beheer uitteoefen oor die potensiële invloed van enige eksperimentele veranderlike op die resultate.

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AN ALPHABETICAL LIST OF ALL ABBREVIATIONS USED IN THE THESIS

ACh	= Acetylcholine
ACTH	= Adrenocorticotropic hormone
ADR	= Adverse drug reactions
AIDS	= Acquired Immune Deficiency Syndrome
EtOH	= Alcohol
SNCA	= Alpha synuclein
AD	= Alzheimer's disease
Aβ	= Amyloid beta
ALS	= Amyotrophic lateral sclerosis
ANOVA	= Analysis of variance
ATC	= Anatomical therapeutic chemical
AVP	= Arginine vasopressin
BBB	= Blood-brain barrier
BDNF	= Brain derived neurotrophic factor
COMT	= Catechol-*o*-methyltransferase
CNS	= Central nervous system
CSF	= Cerebrospinal fluid
CJD	= Creutzfeldt-Jakob disease
cAMP	= Cyclic AMP
CNS	= Central Nervous System
z	= Charge
CRF	= Corticotropin-releasing factor
DLB	= Dementia with Lewy bodies
DAB	= Diaminobenzidine
DA	= Dopamine
DAT	= Dopamine transporter
DIGE	= Differential in-gel electrophoresis
DRP	= Drug-induced parkinsonism
ESI	= Electrospray ionization
ER	= Endoplasmic reticulum
ELISA	= Enzyme-linked immunosorbent assays
EST	= Expressed sequence tag
FTD	= Frontotemporal dementia
GDNF	= Glial Derived Neurotrophic Factor

GPe	= Globus Pallidus externa
GPi	= Globus Pallidus interna
GCs	= Glucocorticoids
GH	= Growth hormone
HSP	= Heat shock proteins
HDL	= High-density lipoprotein
HPLC	= High-performance liquid chromatography
HVA	= Homovanilic acid
HRT	= Hormone replacement therapy
HMG-CoA	= 3-hydroxy-3-methylglutaryl coenzyme A
HPA	= Hypothalamic-pituitary-adrenal
IPG	= Immobilized pH gradient
I.P.	= Intraperitoneal
ICAT	= Isotope coded affinity tag
ICPL	= Isotope coded protein label
IEF	= Isoelectric focusing
pI	= Isoelectric point
iTRAQ	= Isobaric Tagging for Relative and Absolute protein Quantification
kDa	= KiloDalton
LB	= Lewy bodies
L-DOPA	= Levodopa
LN	= Lewy neurites
LC	= Liquid chromatography
LDL	= Low-density lipoprotein
MassSpect	= Mass Spectrometry
MS	= Maternal separation
MALDI	= Matrix-assisted laser desorption/ionization
m	= Mass
MFB	= Medial forebrain bundle
MECT	= Metal element chelated tags
MPTP	= 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine
mtDNA	= Mitochondrial DNA
MAO	= Monoamine oxidase
MudPIT	= Multidimensional protein identification technology
NGF	= Nerve Growth Factor

NFL	= Neurofibrillary tangles
NT-3	= Neurotrophin-3
NT-4/5	= Neurotrophin-4/5
NSDA	= Nigrostriatal dopaminergic
NA	= Noradrenergic
OC	= Oral contraceptive
PVN	= Paraventricular nucleus
PD	= Parkinson’s disease
PBS	= Phosphate buffered saline
PEG	= Polyethylene glycol
PND	= Postnatal day
PRS	= Prenatal restraint stress
Prob	= Probenecid
POMC	= Pro-opiomelanocortin
ROS	= Reactive oxygen species
RES	= Reserpine
STD	= Saturation transfer difference
STN	= Subthalamic nucleus
SDS	= Sodium dodecyl sulphate
SD	= Sprague-Dawley
SILAC	= Stable isotope labelling by amino acids in cell culture
SEM	= Standard error of mean
SOD1	= Superoxide dismutase 1
SELDI	= Surface-enhanced laser desorption/ionisation
SD	= Standard deviation
SN	= Substantia nigra
SNpc	= Substantia nigra pars compacta
SNpr	= Substantia nigra pars reticulate
SNzn	= Substantia nigra zona compacta
SN	= Subthalamic nucleus
ST	= Striatum
STN	= Subthalamic nucleus
SAM	= Sympathetic-adrenal-medullary
TOF-MS	= Time-of-flight MassSpect
2-D PAGE	= Two-dimensional polyacrylamide gel electrophoresis
TH	= Tyrosine hydroxylase

CoQ_{10}	= Ubiquinone
UCHL1	= Ubiquitin carboxy-terminal hydroxylase L-1
UPS	= Ubiquitin proteasome system
VTA	= Ventral tegmental area
WW	= Wet weight
5-HT	= 5-hydroxytryptamine
6-OHDA	= 6-hydroxydopamine

Chapter 5: Drug-regime abbreviations

P + V	= Placebo + rotenone
P + R	= Placebo + rotenone
S + V	= Statins + vehicle
S + R	= Statins + ronenone

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CHAPTER 1

PARKINSON'S DISEASE – AN OVERVIEW

1. Eponym Parkinson's disease: A brief introduction to the disease

Parkinson's disease (PD) is a commonly occurring neurodegenerative disease that was first described as *Paralysis agitans* by the English physician Dr. James Parkinson (for his photograph, see Fig. 1, the image on the left) in his essay, entitled *An essay on the shaking palsy*, published in 1817. This provided the classical characteristics namely bradykinesia, tremor and rigidity that PD is clinically recognized by. Postural instability was added later by the French physician Jean-Martin Charcot (for a photograph of him, see Fig. 1, the image on the right). Together they set the core symptoms that describe the disease. However, since its original description, the clinical conception of the disease has undergone a dramatic transformation in that it is now increasingly recognized as being a more complex syndrome than originally thought, involving both motor and non-motor systems.

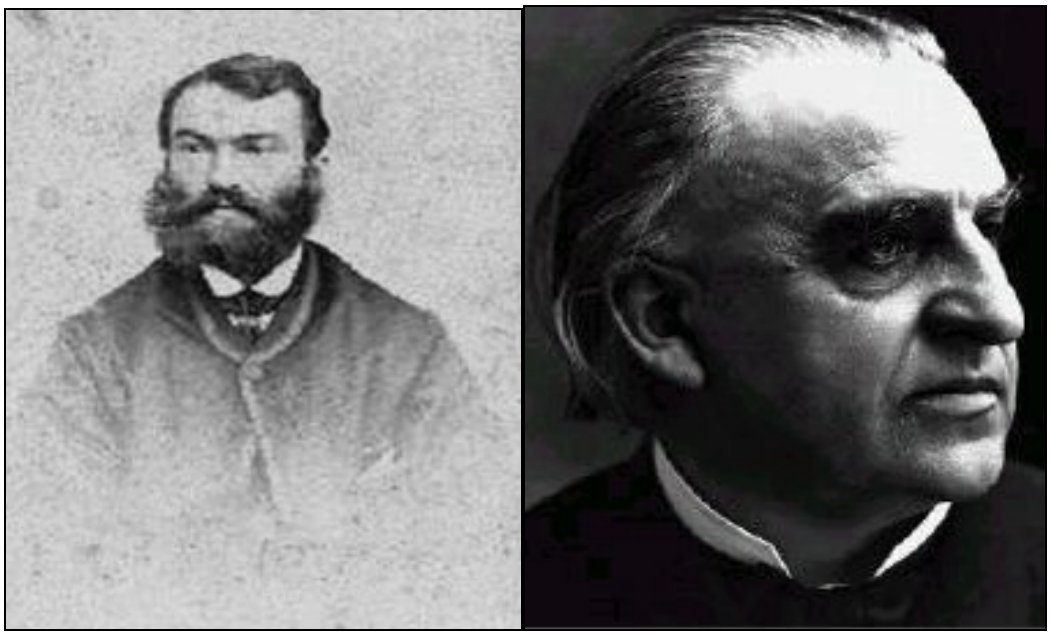

Fig. 1. Photographs of Dr. James Parkinson (1755-1825) on the left and of Prof. Jean-Martin Charcot (1825-1893) on the right.

2. Socio-economic implications of Parkinson's disease

Due to the ever-increasing proportion of the populations of developed countries that comprise of elderly individuals and the recognized relation between ageing and the development of parkinsonism, PD and other related neurodegenerative diseases, place a growing economic

burden on the health care systems of many countries. It has been estimated that caring for PD patients may cost the U.K. Health and Social services £380m per annum, with half the sum that is spent on placing patients in nursing homes and other institutions of care (The Parkinson's Disease Society, 1994). In the same document, it is estimated that the annual cost of the disease per individual to be in the range of £42,000. However, this does not take into account the indirect socio-economic costs associated with the disease, such as loss of productivity. A further difficulty in accurately estimating the costs involved is that the cost per patient is not linearly distributed throughout the course of the disease. During the early phases of the disease, when the patient is still independent and needs a relatively simple drug regimen, less money is spent compared to the later stages of the illness, when more complex and expensive drug regimens are required (Dodel *et al*., 1994).

3. The epidemiology of Parkinson's disease: How common is the disease?

3.1. Age of onset

Epidemiological studies suggest PD to be the second most common neurodegenerative disorder after Alzheimer's disease (AD) (Hague *et al*., 2005), affecting ~2% of the population over the age of 60 years. PD cases are far more unusual in people under the age of 40, and usually these so-called juvenile onset types associate with a clear genetic origin, while non-genetic disease types are principally considered a disease of the elderly. The average age of onset for the sporadic, non-genetic form of PD is approximately 55-60 years, with the rate of PD rising sharply after the fifth decade. This observation was epidemiologically confirmed by Hoehn and Yahr (1967) who revealed that 2/3 of the patients included in their study were between the ages of 50 and 69 when first diagnosed with PD. Only a few other incidence studies on PD have been published, and the Hoehn and Yahr estimations for distribution of age at onset seem to be relevant even today. However, it remains controversial whether there is a progressive rise in PD cases during the later stages of life or if an incidence decline can be seen beyond the 6th decade of life (Mayeux *et al*., 1995; Morens *et al*., 1996; Hofman *et al*., 1989).

3.2. Disease duration in years

In one long-term investigation regarding the distribution of disease duration (Sutcliffe & Meara, 1995), the number of years that patients had been affected by PD ranged from 0 to 42, with a median of 6 years. Only 8% of the patients included in this study had been affected by PD for longer than 20 years.

The Hoehn & Yahr scale (Hoehn & Yahr, 1967) was introduced as a stage-based rating scale of disease severity. The scale comprises of 5 stages, ranging from unilateral disease (stage I) to the patient being confined to bed or a wheelchair (stage V). The authors suggested

that the median duration of illness since onset of the disease was 3 years for stage I and 14 years for stage V. The scale was modified with the introduction of Levodopa into clinical practice that remains the "gold standard" for pharmacologically treating the disease. It was suggested that the duration of each stage should be prolonged by 3-5 years, when patients receive L-DOPA therapy (Hoehn, 1987).

3.3. The correlation between disease prevalence and geographical distribution

Most European and U.S. studies report a prevalence rate of 0.1-0.2%, although a slightly higher figure (0.3%) has also been reported (Aquilonius & Hartvig, 1986). The highest prevalence reported for the disease is for Europe and North America, while this is lower in Asian countries (0.05%). The widespread prevalence rate for China has been estimated to be at 1,700/1,000,000 cases for all people aged ≥65 years (Zhang *et al*., 2005a). Africa seemingly has the lowest prevalence, although it may be argued that the disease often goes undiagnosed in some underdeveloped countries where patients lack access to sophisticated health services (Li *et al*., 1985; Schoenberg *et al*., 1988; Okada *et al*., 1990).

It has been argued that prevalence rates may be affected by socio-economic differences that stem from using "survival" as the sole outcome measure, and problems relating to the implementation and execution of population surveys (Bermejo *et al*., 2001). Therefore, the incidence rates reported for the USA (Rajput, 1984) of 20.5 per 100,000 people, may be a more reliable indicator of the extent of the disease.

3.4. Racial and ethnic differences in Parkinson's disease prevalence

It should be noted that although data trends (as discussed in the section above) suggest the incidence of PD to vary by race/ethnicity (Van Den Eeden *et al*., 2003), published reports often contradict each other. For example, some authors suggest that disease prevalence is lower amongst Blacks compared to non-Hispanic Whites (Marttila & Rinne, 1981; Richards & Chaudhuri, 1996; Kurtzke & Goldberg, 1988; Lilienfeld *et al*., 1990; Schoenberg *et al*., 1985; Van Den Eeden *et al*., 2003), while another study found a higher incidence for PD amongst a Black population (Mayeux *et al*., 1995). Other figures report increasing rates in Blacks (10.2), among Asians (11.3), and non-Hispanic Caucasians (13.6), with the highest occurrence found in Whites of Hispanic descent (16.6) (Van Den Eeden *et al*., 2003). It remains unclear whether methodological differences, such as variation in population characteristics and exposures, case-finding methods and limitations in denominator accuracy may explain this disparity or whether PD is indeed more common among Blacks, but simply underreported. In this regard, the lack of replication for studies suggesting a higher prevalence rate amongst Blacks may be due to inherent methodological errors of the studies. For example, it has been postulated that the

difference between Whites and Blacks for PD prevalence may rather be explained by the discrepancy between the population groups in terms of access to medical care (Schoenberg *et al*., 1985; Mayeux *et al*., 1995).

3.5. Gender variance in Parkinson's disease incidence

Gender differences in the basic disease characteristics, the pattern of motor deterioration and nigrostriatal degeneration have been reported in a number of studies. Epidemiological studies report that the prevalence of PD in men range from 1.5 to twice that found in women (Van Den Eeden *et al*., 2003; de Lau *et al*., 2004; Wooten *et al*., 2004; Schrag *et al*., 2000; Claveria *et al*., 2002; Benito-Leon *et al*., 2003). In addition, a higher incidence rate for the disease had been reported in men (19.0 per 100,000) than in women (9.9 per 100,000) (Van Den Eeden *et al*., 2003). The age of disease onset for women is also slightly later in women than in men (Twelves *et al*., 2003).

It has been postulated that the reported gender-based variance could be related to the difference in circulating reproductive hormones, most notably estrogens, with several findings seeming to support a protective role played by estrogen towards developing PD. Possible gender variance in PD remains a rigorously debated topic, This issue is more comprehensively discussed in chapter 2 of the Thesis.

4. Current understanding of Parkinson's disease aetiology

In the vast majority of PD cases that take on a sporadic appearance, the true aetiology behind the onset and progression of the disease remains completely unknown, and its elucidation remains one of the major challenges of neuroscience. However, in order to comprehend the full extent of the pathology of the disease, one should first consider the mechanics of the intact nervous system, particularly the mechanisms responsible for controlling motor function, after which an assessment can be made of how patho-mechanisms could impact on normal function.

4.1. Motor-related features of Parkinson's disease

4.1.1. The organisation of the intact motor system

Motor behaviour is the product of a complex orchestration of functions induced by the extrapyramidal system. This system consists of a number of interrelated structures that form multiple parallel loops that function to control normal voluntary motor activity (Nyholm, 2003). It is also involved in regulating complex non-motor behaviour (Alexander & Crutcher, 1990). Specific structures include the cortex; striatum (consisting of large subnuclei, including the putamen, which, in turn consists of the external and internal pallidum segments and the ventral pallidum); subthalamic nucleus (STN; consisting of the nucleus caudatus and nucleus

accumbens); globus pallidum and thalamus (commonly referred to as the cortico-striato-pallido-thalamocortical circuit); and the nigral substance (pars reticulata and pars compacta, with the latter containing dopaminergic (DA-ergic) neurons). These subnuclei are intrinsically located in the forebrain and midbrain and relate at a structural and functional level.

The cerebellum also forms a prominent component of this motor-function system, while the basal ganglia plays a key facilitative role to accurately select, prepare and execute normal, everyday movements (Albin *et al*., 1989; DeLong, 1990; Obeso *et al*., 2000).

DA-ergic projections originating in the Substantia Nigra pars compacta (SNpc) terminate on dendrites of the medium-sized spiny neurons in the striatum. These terminating neurons are located in two nucleic zones of the striatum, the caudate nucleus and the putamen (Kotter, 1994). In addition, at cytoarchitectorally, morphologically and neurochemically distinct cell groups, interneurons locate to the striatum (DiFiglia *et al*., 1976; Gerfen, 2004). These two classes of cells differ hugely in terms of relative abundance, with medium-sized spiny neurons representing approximately 95-97% of the total population, while the interneurons contribute merely 3-5% to total neuronal content (Gerfen, 2004).

The various input and output structures of the striatum have vast interconnections (Gerfen, 2004), connecting the direct and indirect pathways of the basal ganglia (Bolam *et al*., 2000) with multiple feedback and feed-forward loops that add to the stability of the basal ganglia network (Olanow *et al*., 2006). The striatum acts as an 'input structure' to govern basal ganglia function (Parent *et al*., 1995; Parent & Hazrati, 1995; Gerfen & Wilson, 1996; Wise *et al*., 1996) by receiving and converging external glutamatergic inputs stemming from the cerebral cortex, thalamus, and limbic structures. Moreover, frontal-cortical-intralaminar-thalamic as well as brainstem nuclei also target the STN, to provide additional input stimuli processed by the basal ganglia (Gerfen, 2004). In addition, the DA-ergic and serotoneric systems (that originates from the raphe nuclei) also modulate the nature of this input.

Together, the globus pallidus pars interna (GPi) and the SNpr provide the main 'output' nuclei of the basal ganglia, by projecting inhibitory γ-aminobutyric acid (GABA)-ergic somatotopical connections to the various thalamic nuclei, colliculi superior and brainstem reticular formations. In addition, the basal ganglia receives 'external' information from every region of the cerebral cortex, midline, intralaminar thalamic nuclei and limbic structures, including the amygdala and hippocampus. Additionally, serotonergic inputs from the raphe nuclei and cholinergic fibers from the peduncopontine nucleus project to parts of the basal ganglia. The basal ganglia-thalamocortical system plays a prominent role in the development and fine-tuning of various processes, most notably those associated with learning, that are essential for producing certain motor as well as non-motor behaviour. For establishing these

processes, behavioral reinforcement is essential, for which the DA-ergic system plays a critical role.

As stated, the cerebellar circuit plays a prominent complimentary role, especially in establishing the exact timing with which motor-behaviour is executed. Once the learning phase of a movement has surpassed, behavioral programs are automated by the basal ganglia, while the cerebellum continues to fine-tune output in relation to sensory feedback. The final behavioral program with which a specific action is executed by is the product of deliberate choices between movements with different probabilistic outcomes by the basal ganglia and cerebellum.

DA fulfils an integral role in stabilizing the basal ganglia network by modulating glutamate-induced excitability of striatal neurons. The DA receptors are divided into 2 classes: those that are D_1-like and the D_2-like. While the D_3 and D_4 receptor subtypes relate to the D_2 family, the D_5 subtype is similar to D_1-like (Sibley *et al*., 1993). Striatal D_1 receptors mainly synapse post-synaptically with neurons located in the stratum (the direct pathway), where they couple to a specific G-protein that activates adenylate cyclase, the enzyme that converts ATP to cyclic AMP (Okada *et al*., 1994). D_2-like receptors are found in high concentrations within the caudate and putamen (Kandel *et al*., 2000) that forms part of the indirect projection pathway (Gerfen *et al*., 1990) to primarily inhibit adenylate cyclase. DA exerts both exertive and inhibiting effects on the D_1 and D_2-like DA receptors of the striatum (Lee *et al*., 2005; West & Grace, 2002). DA's excitatory effect takes place via its transmission through the direct D_1-mediated pathway, exerting an inhibitory effect that is mediated by the indirect D_2-mediated pathway (Clark & White, 1987) on cortical motor areas.

The consistent striatal DA-ergic stimulation which characterize the nigrostriatal DA-ergic neurons serve to regulate glutamatergic input from the cortex to the direct and indirect pathway that extend from here (Levy *et al*., 1997). GABA-ergic efferents project both directly as well as indirectly to the internal segment of the GPi and the SNpr to form the two major output nuclei of the basal ganglia (Chase *et al*., 1998). Whereas basal ganglia output directs to several thalamic and brainstem nuclei with a direct, monosynaptic pathway leading from the striatum to the GPi, the indirect pathway includes synapses formed in the external segment of the globus pallidus (GPe) and the SN (Nyholm, 2003).

4.1.2. Motor-related clinical features of Parkinson's disease

Throughout the 19th century, clinical signs indicating an adverse effect exerted on the voluntary motor system was established as the symptoms that entail the PD syndrome. However, the cardinal pathological feature responsible for this functional impairment, namely a functional disturbance of the DA-ergic neuronal system due to the chronic, progressive degeneration of

mesencephalic DA-ergic neurons resident to the SNpc (Nakano & Hirano, 1984; Perry *et al*., 1985; Rinne *et al*., 1989) was only established much later.

The syndrome comprises of a reduced speed with which patients execute movements (*bradykinesia*), scarcity of spontaneous movements (*hypokinesia*), tremor, rigidity and postural instability (LeWitt & Oertel, 1995). Hypokinesia has often mistakenly been regarded as "weakness", exemplified by the term 'Paralysis Agitans', an alternative term for describing PD symptoms.

A brief definition and description will now be given of some of the common features that make up the clinical phenotype of PD.

Dyskinesias can be consistent, or present episodically as abnormal movements. They essentially comprise of movements that had been slowed down considerably compared to the speed with which they were performed in the absence of disease (*bradykinesia*). Additional features include an inhibition of spontaneous movements (*akinesia*), or the excessive display of spontaneous movements (*hyperkinesias*).

Tremor is described clinically as rhythmic, involuntary, oscillatory movements, characterized by fixed amplitude and extended duration. Although tremor is the best-recognized symptom of PD, only 60-70% PD patients ever suffer from it. Its manifestation may be due to a disinhibition of the thalamus that stems from an underlying striatal-DA deficiency. Evidence for this derives from the finding that stereotactic lesions made at this level provide symptomatic relief (Wilms *et al*., 1999).

Rigidity is an additional clinical hallmark in parkinsonism. It is believed that enhanced long-latency stretch reflex underlies this clinical phenomenon, resulting in increased resistance. The cogwheel phenomenon is seen during clinical investigation of PD sufferers when patients complain of experiencing a sense of stiffness during movements carried out on the patient by the physician that entails passive flexion/extension (Jankovic, 1992). This symptom may co-manifest with a loss of dexterity, micrographia, hypophonia, and a shuffling small-stepped gait. Often at times, this may co-manifest with bradykinesia.

Postural imbalance is the least specific, though often the most disabling parkinsonian symptom, producing the stooped posture, pro- and retropulsion by which the disease is so often recognized by (Jankovic, 1992). It is likely that parkinsonian gait and postural instability result from a combination of bradykinesia, rigidity and loss of proprioceptive reflexes.

Additional symptoms of the disease include ocular (specifically, impaired saccadic and smooth pursuit eye movements) and speech abnormalities. These symptoms may express in consequence to a dysfunction of the striatal DA-system, particularly of the parallel ocular and phonological circuits based within the extrapyramidal system (Marsden & Obeso, 1994).

In addition, the clinical phenotype that associates with early-onset recessive parkinsonism (that often have an underlying genetic mutation), often includes dystonia at onset and hyperreflexia, with the disease progressing relatively slower than with the sporadic type (Gasser, 2005). However, it should be noted that only a weak correlation between clinical manifestation and type of genetic mutation has been described. However, the available case series indicate that patients with point mutations tend to be more mildly affected than patients with deletions (Lohmann *et al*., 2003).

4.1.3. Patho-mechanisms involved in the manifestation of motor-related features of Parkinson's disease

Although both DA-ergic and non-DA-ergic nuclei belonging to the nigrostriatal pathway are adversely affected in PD (Dauer & Przedborski, 2003), the destruction of DA-ergic neuronal afferents in the SN, resulting in reduced DA levels in the corpus striatum (Ascherio *et al*., 2003) is widely accepted as being PD's cardinal pathological hallmark. In addition, the fact that various regions of the cerebral cortex are innervated by DA-ergic neurons entails that the functions associated with these areas may also be altered by the disease (Thierry *et al*., 1973; Thierry *et al*., 1974; Divac *et al*., 1978). DA fibre terminals are predominantly confined to the deeper layers of the frontal, cingular, and entorhinal cortical areas. In contrast, the noradrenergic (NA) axonal terminals widely disperse throughout this anatomical region (Hökfelt *et al*., 1974; Berger *et al*., 1976).

Various compensatory mechanisms are triggered in response to reduced DA production. These become prominent during the initial stages of the disease. Under these conditions, robust DA reuptake mechanisms are in place for sufficiently maintaining striatal and synaptic DA concentrations, thereby exposing synaptic and extrasynaptic striatal DA receptors to DA concentrations that undergo relatively few fluctuations. In addition, increased activity by the surviving DA neurons, downregulation of DA transporters (DAT), increased sensitivity of postsynaptic DA receptors, and modified firing rates of the basal ganglia neurons may occur. However, in the long-term these compensations fail, eventually leading to a loss of cortico-striatal functions that would normally be modulated by DA (Calabresi, 1993). In addition, the ability of striatal neurons to undergo long-term potentiation and long-term depression become impaired (Calabresi, 1993; Centonze *et al*., 1999), while the firing frequencies and firing patterns of neuronal populations in the GPe, GPi and STN are distorted (Albin *et al*., 1989; DeLong, 1990; Obeso *et al*., 2000; Raz *et al*., 2000). Furthermore, DA depletion results in the desynchronized firing of pallidal neurons in response to peripheral stimuli, leading to an impaired ability to accurately select an intended movement (Filion *et al*., 1988; Nini *et al*., 1995).

A significant reduction in the number of postsynaptic DA receptors can also induce PD, leading to a functionally misbalancing striatal output structures, thereby producing the same overt signs and symptoms as other forms of spontaneous-onset PD.

4.5. Non-motor features of Parkinson's disease

New insights gained into the pathological processes underlying the disease, have led to the realisation that PD is a syndrome that stems from the degeneration of a multitude of physiological systems. Extensive neuronal connections exist between the motor- and the limbic system for relaying and processing emotive and cognitive-associated information that the motor system reacts on. The mechanisms of the intact interrelation between the cortical, limbic and motor system will now be discussed, followed by the clinical features associated with its diseased-state, and then a discussion on the patho-mechanisms that might be responsible for these clinical manifestations.

4.5.1. The organisation of the intact cortical and limbic systems and their relation to the motor system

The human cerebral cortex consists of an extensive neocortical territory and a smaller allocortical region. The neocortex is partitioned into primary fields to allow for motor functions and for processing sensory information reaching the brain from the sensory organs. Each neocortical primary sensory field receives somatosensory, visual and auditory information, and is surrounded by unimodal secondary areas, each connected to additional, unimodal as well as heteromodal association areas (Braak *et al*., 1998; Zilles, 1990). Sensory (auditory and visual) and somatosensory exteroceptive input reach their matrix of related association areas within the neocortex via the primary fields and secondary areas. Once it has reached its destination, data are conveyed by means of long cortico-cortical pathways towards the extensive region that makes up the prefrontal cortex. Short pathways lead away from this region towards subordinate fields through premotor areas, into the primary motor field. Incorporated into these pathways are the striatal and cerebellar loops that guide the information back to the primary motor field. In this way, large portions of the basal ganglia, the lower brain stem, and the cerebellum all participate to regulate cortical output.

The prefrontal cortex is constantly inundated by a large supply of vast amounts of largely irrelevant exteroceptive stimuli arriving from the sensory association areas. Neocortical data flow into the limbic loop via two entry ports. If the receiver regards the data as being useful, this is filtered out of the main stream arriving via many intermediate neocortical relay stations, eventually converging on the temporal pro-neocortex and periallocortical transentorhinal region, located in the entorhinal region and amygdala, situated at the first entry point to the limbic

system. The second port of entry for neocortical data entering the limbic loop comprises of the lateral nucleus of the amygdala. Under non-pathological conditions, both the central amygdala nucleus and the bed nucleus of the stria terminalis exert an influence over the autonomic and neurosecretory nuclei of the hypothalamus. In addition, the central nucleus of the amygdala also directs the processing of viscero-sensory data and controls all viscero-motor areas of the brainstem and spinal cord.

The efferent trunk of the limbic loop consist of projections running from the entorhinal region, amygdala and hippocampal formation, exerting an important influence on the prefrontal cortex. Finally, the central nucleus of the amygdala exerts critical influences on all non-thalamic nuclei with diffusely connecting projections extending to the cerebral cortex and many other structures of the central nervous system (CNS). These projecting nuclei that include the cholinergic magnocellular nuclei of the basal forebrain, the GABAergic tuberomamillary nucleus of the hypothalamus, the serotonergic raphe nuclei, the DA-ergic nuclei of the ventral tegmentum and the noradrenergic locus coeruleus, undergo severe pathological changes during the progression of PD.

The allocortex essentially comprises of the hippocampal formation and the presubicular and entorhinal regions, while the subcortical amygdala is a closely related structure. The entorhinal territory and the lateral nucleus of the amygdala act as a gateway to either allow or forbid information transfer. Fig. 3 below shows the relation between these regions.

The centres of the limbic loop subsequently dispatch their efferents in the direction of the neocortex, specifically towards the prefrontal areas (Holstege, 1996; Mesulam, 1998; Nieuwenhuys, 1996). From the above description, it is therefore clear that these efferents exert considerable influence on the neocortex, forming a constituent component of the motor system. As can be viewed from Fig. 4, all these centres are intricately connected. The limbic loop performs a crucial role in maintaining emotional equilibrium, the individual's ability to learn, and memory function. Simultaneous, it affects motor function, such as the influence the limbic system has on the prefrontal cortex. This may serve to explain why motor activity can perhaps be regarded as reflecting the individual's emotional state.

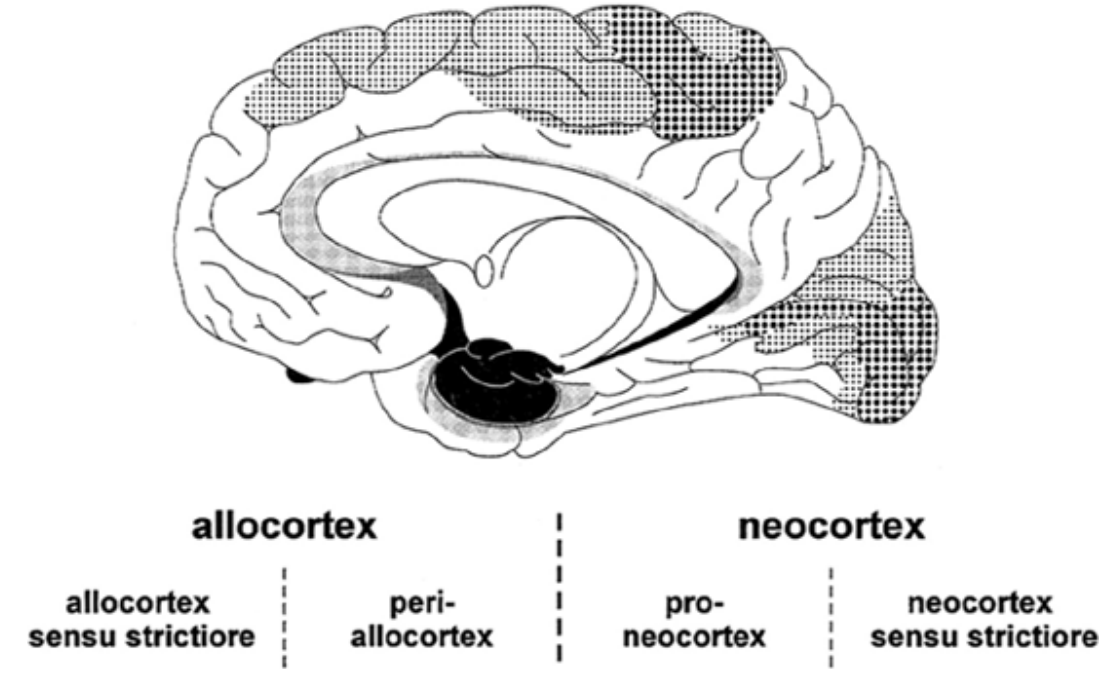

Fig. 2. The relation between the limbic system and the neocortex. The figure shows the frontal, parietal, occipital and temporal neocortices, with each comprising of a primary field (indicated by the large dots), a belt of secondary fields (indicated by the small dots), and related higher-order processing areas (the white areas). Figure taken (with permission) from Braak *et al*. (2003).

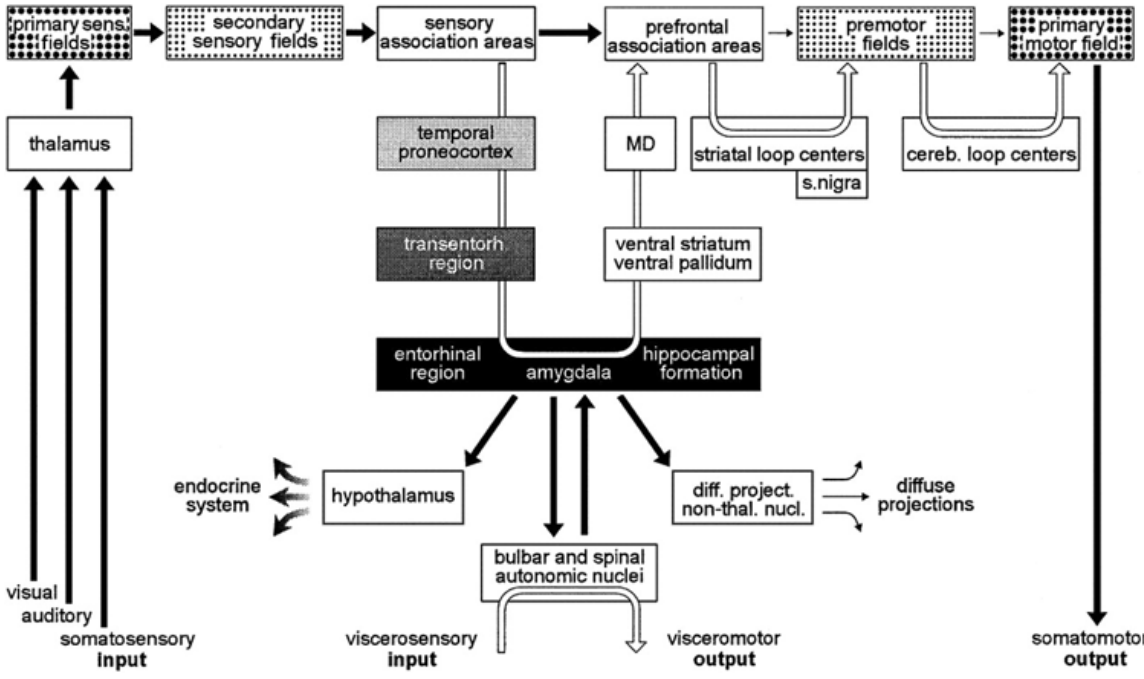

Fig. 3. A schematic representation of the limbic and motor system that facilitates the perception of intimate connections and the tight relations between these two systems. Figure taken (with permission) from Braak *et al*. (2003).

4.2.2. Non-motor clinical features of Parkinson's disease

Although PD can manifest purely motorically, motor symptoms often co-expresses with complex, non-motor behavioral abnormalities. These may include cognitive, emotional, neuropsychiatric, motivational and autonomic dysfunction. In addition, a disturbed nocturnal sleep pattern with excessive daytime sleepiness is frequently co-diagnosed along with PD, the occurrence of which seemingly increases with advancing disease (Askenasy, 1993; Tandberg *et al*., 1999). These non-motor abnormalities may occur intrinsically to the disease or extrinsically due to dopaminomimetic and/or neurosurgical therapeutic interventions (van de Berg *et al*., 2007). Whereas some of these complications are possibly induced by dopaminomimetic therapy, others might be linked to disease progression instead (Nyholm, 2003).

Therefore, the primary motor dysfunctions that characterize PD are frequently accompanied by cognitive impairments, such as memory deficits, decreased conceptual ability, behavioral deregulation and defective visuo-spatial functions (Mizoguchi *et al*., 2004; Sullivan & Gratton, 1998). It is now a well established that patients with PD also have markedly impaired olfactory function (Doty *et al*., 1988; Hawkes, 2003), with this clinical feature corresponding well to neuropathological findings, such as the presence of Lewy bodies (LBs) found in the anterior olfactory nucleus (Hawkes, 2003).

Impulse control disorders (ICDs) can seemingly also occur in a subset of patients with PD. This spectrum of disorders, which is characterized by excessive or poorly controlled preoccupations, urges, or behaviours that includes punding, pathologic gambling and hypersexuality, as well as compulsive shopping and binge eating. However, in a small percentage of patients, these behavioral abnormalities associate with the overuse of DA replacement therapy (DRT) and are referred to as a homeostatic hedonistic dysregulation, or as DA dysregulation syndrome (DDS) (Giovannoni *et al*., 2000).

It is frequently reported that patients who suffer from abnormalities that affect the motor-system, also suffer from a range of psychiatric disorders (i.e. psychosis, affective disorders or somatization disorders). It is generally accepted that the expression of motor symptoms is influenced by the patient's state of mood. Emotionally straining situations, and those evoking anxiety or general stress tend to predispose the manifestation of dyskinesias, while their prevalence seems to decrease, or even completely fail to manifest during relaxed periods.

Clinical evidence suggests that stress can increase the severity of PD symptoms (Smith *et al*., 2002). For example, reactions such as anxiety and anger, as responses to stressful situations may effectively worsen patient's tremor (Schwab & Zieper, 1965).

Due to a severe DA deficiency existing in the caudate and accumbens nuclei, psychomotor retardation and apathy are frequently seen in PD patients. The affective and emotional deregulation resulting in depression-like symptoms in the patient, leading to further social isolation and social stress. It has been suggested that difficulties in coping with the physical restraints imposed by the disease and accepting the invalidating condition that is PD, as well as concomitant fatigue and physical pain may play a defining role in this symptom manifesting (Langer, 1994).

In addition, other psychological elements, such as feelings of guilt, loss of self-respect, fear of the inability to continue to partake in occupational and recreational activities, as well as fearing the loss of independence, may also contribute to the severity with which the disease manifests in patients (Dakof & Mendelsohn, 1989). It has been suggested that these psycho-social factors may be important in the development of PD-related depression, stress and social isolation (Seiler *et al*., 1992).

4.3. Cellular and molecular features of Parkinson's disease

The molecular pathways leading to PD's pathological picture remain complex. A number of patho-mechanisms other than a depletion of DA have been proposed to play a part in the etiogenesis of the disease, including the role played by oxidative stress, glutamate-induced excitotoxicity, depleted levels of endogenous antioxidants, reduced expression of trophic factors, inflammatory processes and a dysfunctional protein degradation (for a comprehensive recent review, see Schapira, 2006). Since the themes of protein aggregation, mitochondrial dysfunction and proteasomal stress continue to be observed in many models of PD as well as in clinical studies, it is conceivable that these factors may play central parts at some stage of PD pathogenesis, and novel therapeutic strategies are aimed at.

4.3.1. Misfolded protein aggregation as a contributing cause of Parkinson's disease

Presently it is unclear whether accumulation of misfolded proteins leading to PD's characteristic cytoplasmic inclusions, serve a toxic or a neuroprotective purpose (Thomas & Beal, 2007). While Sir James Parkinson originally described the clinical characteristics of PD in a monograph published in 1913, Friederich Lewy was the first to observe neuronal cell loss in the nucleus basalis of Meynert of patients with PD (Greenberg *et al*., 1985). In addition, he is given credit for being the first to describe the presence of LBs. These round, eosinophilic inclusions display a pale halo, and are found abundantly in the neuronal cytoplasm of the SN of PD brains. Their

microscopic observation have also been made in surviving DA-ergic neurons of other brain areas not classically associated with the disease, such as the cortex and the magnocellular basal forebrain nuclei (Braak *et al*., 1995). It should however be noted that LBs also appear in the brain of certain neurologically intact individuals as well as several other completely unrelated neuropathological conditions, such as sub-acute sclerosing panencephalitis and Hallervorden-Spatz disease (Calne, 2004), thereby diminishing the previously held edifice concerning the pathological significance of LBs. The biochemical constituents of LBs remained unknown until the publication of two studies that brought the alpha-synuclein (SNCA) protein to centre stage. Polymeropoulos *et al*. (1997) reported finding a missense mutation in the gene encoding for SNCA on chromosome 4 in a family that displayed juvenile PD. In addition, using post-mortem PD brains, antibodies against SNCA strongly stained for LBs and LNs (Spillantini *et al*., 1997).

Morphological differences between LBs and LNs have been detected based on the brain region where they were detected. For example, LBs observed in the brainstem region comprise of an eosinophilic hyaline core and a pale-staining peripheral halo. In addition, LBs and neuronal loss is frequently accompanied by macrophages filled with pigment and gliosis when present in the pigmented parabrachialand paranigral nuclei of the ventral tegmentum, locus ceruleus, raphe nuclei of the ventral tegmentum, locus ceruleus, raphe nuclei, dorsal motor nucleus of the vagal nerve, and the endoplasmic reticular (ER) formation (Braak *et al*., 2003).

It was revealed through microscopic investigations that LBs are present in monoamine producing cells. Thereby LB-presence has effectively become the *sine qua non* for confirming a non-differential diagnosis of PD (Calne, 2005), since these abnormal protein aggregations have been detected in both sporadic and familial forms of the disease (Mezey *et al*., 1998; Spillantini *et al*., 1998). Furthermore, knowledge on the existence of a relationship between LB formation and PD pathology also led to discovering that the first pathological changes that precede full disease-pathology, commence in the olfactory bulb, particularly in the anterior olfactory nuclues (Del Tredici *et al*., 2002).

However, in recent times the belief that LBs form the hallmark of the disease has been resisted with the discovery by Rajput *et al.* (1991) that in certain patients who display all the clinical features of PD, neurofibrillary tangles, instead of LBs are present in the SN. However, the reason for the absence of LBs in this subgroup of patients remain elusive.

The disease may be in consequence to changes occurring in the neuronal cytoskeleton that develop in only a few susceptible types of nerve cells. The afflicted neurons eventually develop alterations that can be observed as intracytoplasmic inclusions evolving to become LBs in the perikarya and LNs in the neuronal processes (for a review, see Braak *et al*., 2000). These are

presumably located in portions of axon. Currently it is a matter of debate whether LNs are produced in the dendrites as well.

The major structural components of LBs and LNs are abnormally phosphorylated neurofilaments of the cytoskeleton as well as fibrillar SNCA (Spillantini *et al*., 1998). SNCA is a chaperon protein that attaches to lipid membranes under normal conditions, preferentially localizing in axons and presynaptic terminals of the neurons that are found widely dispersed throughout the brain. It is primarily involved in the maintenance, storage and regulation of DA vesicles at the synaptic terminals (Clayton & George, 1998). During the pathological processes that underlie PD and for reasons that are currently unknown, this presynaptic protein changes its conformation, aggregates and accumulates in the axons and soma of vulnerable neurons. However, whether it is the inclusion body itself, or its principal protein component (SNCA) that is responsible for the effects exerted on the DA-ergic neurons remains an open question. Kramer *et al*. (2007) demonstrated a less prominent role for LBs in neural toxicity, by using a protein aggregate filtration assay to show that abundant presynaptic terminal associated SNCA aggregates promote synaptic pathology and neurodegeneration.

4.3.2. Mitochondrial dysfunction as a contributing cause of Parkinson's disease

Mitochondrial pathology has been suggested to play an underlying role in PD pathogenesis (Silvestri *et al*., 2005; Sim *et al*., 2006). The active compound of MPTP, the neurotoxin MPP^+ shows the capacity to inhibit mitochondrial complex I (Lee & Martens, 1986). This finding supports the suggestion that an energy failure resulting from the inhibition of the mitochondrial respiratory chain is a likely mechanism to result in neuronal death in cases of MPTP-induced Parkinsonism. In addition, a decrease in complex I activity has been reported in biopsies done on skeletal muscle (Schapira *et al*., 1990; Mizuno *et al*., 1989). A reduction of 45% in respiratory chain complex I activity compared to control subjects were also detected in the platelets of PD patients (Parker *et al*., 1989), to indicate that the complex I defect can be present in the peripheral tissue of PD patients as well. In contrast, Mann *et al.* (1992) reported normal platelet and skeletal muscle complex I activities in PD. In addition, respiratory chain complexes I and IV of the lymphocytes of PD patients were found to be impaired compared with age-matched control subjects (Santosh & Pasupathy, 2006). However, in a study performed by Yoshino *et al.* (1992) contrasting results were obtained revealing normal complex I and IV function and a merely slight decrease in the activity of complex II, when lymphocytes of PD patients were analysed.

An elevation of iron has also been observed in the SN with staging of PD, especially in the SN zona compacta (SNzn). The iron has been found present in glia, active microglia, macrophages, oligodendrocytes that lie outside of the degenerated DA neurons and as a mild

halo around LBs and within melanized dopamine neurons of SNzc. The iron in SNpc may induce oxidative stress and thus associate with the reported decreases of glutathione peroxidase activity, reduced glutathione (GSH), mitochondrial complex I activity, calcium binding protein and increased basal lipid peroxidation. To support these claims, Schapira *et al*. (1990) and Mizuno *et al*. (1989) found a marked reduction in complex I activity during postmortem analyses done on the SN of brain samples obtained from PD patients.

What remains uncertain are the mechanisms responsible for these functional defects of mitochondria, and whether these are the primary or secondary cause of the disease. It has been postulated that the respiratory chain defects might be secondary to the effects of environmental toxins that inhibit the respiratory chain and result in increased free radical production (Langston, 1985; Nicklas *et al*., 1985; Nagatsu & Yoshida, 1988). Such an inhibition could ultimately result in nonselective damage incurred to components of the respiratory chain (Dexter *et al*., 1989; Leehey & Boyson, 1991).

Damage sustained at the protein coding level is also a distinct possibility (Ikebe *et al*., 1990). The vast majority of mitochondrial proteins are encoded for within the nuclear DNA. These include numerous components of the electron transport chain, proteins involved in mitochondrial DNA replication, transcription and translation, as well as all matrix, inner and outer membrane proteins. These proteins are transported to their specific mitochondrial compartments by means of signal peptides. Therefore, disruption of the electron transport chain could potentially occur in either mitochondrial or nuclear DNA.

It is clear that further work is required at the molecular level to determine the precise clinical relevance of these molecular disorders. This topic will be returned to in chapter 5 of this Thesis, where it forms a major theme of this particular chapter.

4.3.3. The involvement of the ubiquitin proteasome system (UPS) in Parkinson's disease

Parkin is one of three (the others being DJ-1 and PINK1) mutated genes associated with familial parkinsonism. It is typically found present in autosomal recessive inherited forms of the disease and is implicated in mitochondria and oxidative stress-related survival pathways (Abeliovich and Beal, 2006). It is by now well established that parkin functions as an ubiquitin ligase in the cellular ubiquitination/protein degradation pathway (Shimura *et al*., 2000), by encoding for (at the carboxy terminus) an E3 ubiquitin ligase with the characteristic two Really Interesting New Gene (RING) finger domains separated by an IBR (In Between Ring) domain. This domain is common to other E3 ligases as well. *Parkins's* domain structure suggests it acts as a gatekeeper, to covalently add ubiquitin chains to tag proteins prior to their destruction by proteasomes. It has been demonstrated that the parkin gene not only mediates the well-studied

addition of ubiquitin via lysin48 (K48), which accounts for directing ubiquitinylated proteins for proteasomal degradation, but also via lysin63 (K63). This may play a role in intracellular signaling processes as well as in LB formation (Lim *et al*., 2005), since dardarin, ubiquitin and other proteins involved in ubiquitin metabolism form the major constituents of LBs (van de Berg *et al*., 2007). However, it remains uncertain whether and how the loss of ubiquitin function leads to neurodegeneration. The uncertainty is largely due to inconclusive evidence for a single putative toxic protein responsible in this chain of events, although it has been hypothesized to accumulate due to the lack of parkin in PD patients or parkin knock-out mice (Gasser, 2005).

4.3.4. Microglial activation as a possible contributing cause of Parkinson's disease

Certain infections, including encephalitis lethargica, Creutzfeldt-Jakob disease (CJD), Acquired Immune Deficiency Syndrome (AIDS) and syphilis, present with the same cognitive, behavioral and motor deficits as seen in PD cases. Moreover, these infectious diseases all show infected microglia, a neuropathological feature shared by PD. Under ordinary circumstances, microglia are regarded as the resident immune cells of the CNS (del Rio-Hortega, 1932). Microglia and related perivascular macrophages provide a first line of defense to protect the CNS microenvironment against injury or disease. Their functions range from performing an immunological survey of the brain parenchyma to a neuroprotective role. They do so by monitoring extracellular chemical changes, debris accumulation, pathogen invasion and altered neuronal signalling. In addition, they may play a role during apoptosis and secrete a number of soluble factors, amongst them glial-derived neurotrophic factor that possibly play a role in ensuring neuronal survival (Streit, 2006).

Several lines of clinical and experimental evidence support the hypothesis that glial activation and inflammatory processes play a role in the pathogenesis of PD (Purisai *et al*., 2007). NADPH oxidase is a complex enzyme that is composed of three cytosolic as well as two membrane-bound subunits (Babior, 2004). Microglial activation results in these cytosolic components migrating towards the membrane. Upon reaching their destined location, they bind to the other subunits to assemble active oxidase, which is capable of catalyzing the one-electron reduction of oxygen to superoxide (Purisai *et al*., 2007). Experimental neurotoxins such as 6-OHDA and MPTP possibly exacerbate the neurodegenerative process by activating microglia. Moreover, epidemiological studies report a lower risk for developing PD in patients who regularly use non-steroidal anti-inflammatory drugs (Chen *et al*., 2005). In addition, increased numbers have been found in the SN and striatum of patients with idiopathic PD (McGeer & McGeer, 2004; Ouchi *et al*., 2005), while subsequent to injecting themselves with MPTP, post mortem studies done on the SN of patients who had developed a parkinsonian

syndrome, revealed clusters of reactive microglia based around nerve cells (Langston *et al*., 1999).

The aging brain is characterized by a demonstrable degree of atrophy, presumably due to the loss of neurons and myelinated axons (Conde & Streit, 2006). In contrast, glial cells appear to increase in the aging brain, exhibiting greater immunoreactivity with both astrocytic and microglial markers. During normal aging, microglia undergo structural deterioration, such as abnormalities in microglial cytoplasmic structure, including deramification, spheroid formation, gnarling and fragmentation of processes (Streit *et al*., 2004). Although this may easily be interpreted as normal aging-related neuroinflammation, no definite correlation between ostensible microglial activation and neurodegeneration has thus far been demonstrated, with no correlation found between the location of activated microglia and the location of damaged or lost neurons (Finch *et al*., 2002).

The literature on acute microglial activation with aging does not reveal entirely consistent findings, in large part due to the diversity of injury models, species, anatomic regions, microglial markers and methods of analysis that were used. It is therefore reasonable to assume that there are no obvious aging-related impaired ability of the microglia to respond to CNS injury. However, this statement only applies to experimental animals, primarily rodents, where it is possible to perform time-based studies of acute microglial activation. In human beings, the scenario is likely to be different and far more complicated in consequence to the extended lifespan of the species.

Experimental work performed on rodents suggests that microglia are subject to replicative senescence, such as loss of mitotic ability following repeated rounds of replication. The concept of microglial senescence offers a novel perspective on aging-related neurodegenerative disease, of which PD is a notable example, by regarding neurodegeneration as secondary to microglial degeneration, adversely affecting the viability and self-renewal capacity of microglia. Such attrition of the brain's immune system could contribute to the development of neurodegenerative disease by diminishing glial neuroprotection.

Rodrigues *et al.* (2004) demonstrated that an interaction between astroglial and microglial cells might be involved in incurring damage and in repair-based cascade of events taking place within the ventral tegmental area (VTA) in a unilaterally lesioned 6-OHDA rat model of PD. The Tyrosine hydroxylase (TH) positive DA cells, the glial fibrillary acidic protein (GFAP) immuno-labeled astrocytes and the OX42 immunoreactive microglia were visualized by means of immunohistochemistry and quantified with stereologic methods. Their results include a 46% decrease in the number and density of TH immunoreactive cells in the VTA of 6-OHDA injected rats. GFAP immunohistochemistry also revealed an increased number and density of astroglial cells, with a 6-OHDA dosage-related increase of 154% and 166% compared to the unlesioned

control side. Increased number (76% to control) and density (77% to control) of OX42 microglial labeled profiles and microglial processes (51% to control) were also found in the ipsilateral VTA of the OHDA injected animals that had received a medium dosage. This suggests that the retrograde degeneration of the mesostriatal dopamine pathways induced by a striatal injection of 6-OHDA, leads to astroglial and microglial reactions in the VTA.

Microglia have also been implicated in the production of reactive oxygen species (ROS) and the selective degeneration of nigrostriatal DA-ergic neurons as a consequence of exposure to all the known neurotoxicants used during both *in vitro* and *in vivo* experimental paradigms of PD. This includes systemic exposure to the lipophilic pesticide rotenone (Gao *et al*., 2003; Sherer *et al*., 2003; Ling *et al*., 2004) and MPTP (Gao *et al*., 2003; Sherer *et al*., 2003). Although a single intraperitoneal injection of 10 mg/kg of the herbicide paraquat did not cause a loss of DA-ergic neurons in the mouse SNpc (McCormack *et al*., 2005), it appears to induce microglial activation (Purisai *et al*., 2007). A significant number of cells displaying morphological and immunocytochemical characteristics of activated microglia were already present at 1 day after paraquat administration. Although, the exact relationship between this phenomenon and neurodegeneration is still to be elucidated, intraneuronal deposition of SNCA and the selective degeneration of DA-ergic neurons in the SN, comprising the pathological hallmarks of the disease, are only seen following subsequent exposures to paraquat (Manning-Boð *et al*., 2002; McCormack *et al*., 2002; Thiruchelvam *et al*., 2003; Peng *et al*., 2004). This suggests that the initial exposure to the toxin only predispose DA-ergic cells to degeneration, with the effect coming to a full once the cells have been subjected to a subsequent challenge (McCormack *et al*., 2005). Such a “priming” effect may be highly relevant to PD since it could underlie the mechanism of disease risk factors and help to identify conditions that enhance the vulnerability of DA-ergic cells to degenerative processes.

4.3.5. Genetic mutations as a cause of Parkinson’s disease

The aetiology of PD remains incompletely understood. Although an interaction between genetic and environmental factors is believed to underlie sporadic PD in the vast majority of cases (Sherer *et al*., 2003a), a genetic mutation or loci is regarded as the sole cause for manifesting the disease in a small minority of cases (for a review, see Spacey & Wood, 1999). Approximately 15% of diagnosed cases of PD reveal a positive family history of the disease (Gasser, 2005). Analysis performed in order to link the health status of members of large families and the positional cloning of an increasing number of genes that may be responsible for manifesting monogenic forms of PD, have provided substantial new insights into the pathogenesis underlying the disease. These studies have linked several genes to autosomal recessive forms of PD (Gasser, 2005). Since the discovery of the first PD gene locus in an

Italian family (Polymeropoulos *et al*., 1996), a number of PARK genes (1-8) have been identified to be involved in the development of PD symptoms (Foltynie *et al*., 2002). This finding has led to the nosology of various genetic subtypes of parkinsonism, including mutations of the PARK 1 locus (Singleton *et al*., 2003), which subsequently led to the identification of aggregations of SNCA in PD and the finding that LBs are rich in this protein. SNCA was the first gene to be associated with the aetiology of genetic forms of PD, with the protein believed to play an essential role in synaptic transmission, synaptic vesicle recycling, DA storage and the compartmentalization of neurotransmitters. It also associates with vesicular and membranous structures (Abeliovich *et al*., 2000; Yavich *et al*., 2004; Yavich *et al*., 2006), and therefore a reduced amount of vesicles have been associated with *SNCA* mutations. It has been suggested that unstored DA due to a mutated form of *SNCA* might lead to an increase in oxidative stress to possibly explain the pathogenesis behind PD (Lotharius & Brundin, 2002).

Although the precise relationship that familial linked genes have to the more common sporadic illness remains uncertain, parkinsonism with nigrostriatal DA-ergic degeneration seem to be a common pathogenic mechanism that underlie both disease entities (Hardy *et al*., 2006). In addition, lack of a clear family history of PD in the vast majority of disease-cases seems to rule out a primary genetic defect involving a nuclear gene.

The discovery of a mendelian inheritance pattern that underlies certain forms of PD have allowed the development of novel genetic animal models (using knock-out and knock-down paradigms), providing the basis for improving understanding of the molecular pathogenesis of the disease. It is generally believed that the molecular pathways identified in monogenic cases may provide relevance to possible pathogenic mechanisms implicated in the common sporadic form of the disease. This improved understanding is expected to contribute to the development of novel treatment strategies. More complex genetic models that allow for the interaction of several genetic variants or gene-environment interplay may tease apart the relative contribution each makes towards disease pathology.

An ever-increasing number of novel genes are being discovered, enabling a novel disease classification based on aetiology. This suggests for a high degree of etiological heterogeneity, as it is becomes increasing clear that mutations in different genes might cause the same disease entity at both the clinical and the sub-clinical level. Alternatively, mutations in the same gene may associate with different clinical and pathological outcomes. The various genetic forms of PD will now be discussed.

4.3.5.1. Autosomal recessive forms of Parkinson's disease

Recessive forms of Parkinsonism find their origin in mutated genes that are increasingly recognized as representing an important patho-etiological mechanism in early-onset PD, with

the age of onset usually being prior to 40 years of age. The mutated loci or genes identified thus far include *parkin* (PARK2), PINK1 (PARK6) and DJ-1 (PARK7). The clinical phenotype associated with early-onset recessive parkinsonism often includes additional symptoms, including dystonia at onset, and hyperreflexia, with the disease following a relatively slow progression (Gasser, 2005).

4.3.5.1.1. PARK2 (PARKIN) mutations as a cause of Parkinson's disease

A disease that stems from an autosomal recessive inheritance pattern usually appears in one or more siblings only (a single generation), born from unaffected parents. Among the three autosomal recessive forms of parkinsonism, the version caused by the *parkin* gene are by far the most common (Gasser, 2005). It has been suggested that the source of recurring mutations in *parkin* may stem from mutational hot spots as well as be due to founder effects (Hedrich *et al*., 2004a).

Certain heterozygous mutation carriers identified in large families with *parkin* mutations, presented with symptoms of late-onset PD or minor parkinsonian symptoms, while manifesting mutation carriers have been identified in most mutational screens (Pramstaller *et al*., 2002; Khan *et al*., 2005). However, a similar frequency of heterozygous mutations in the *parkin* gene was found when elderly healthy individuals were compared to a cohort with late-onset typical PD (Lincoln *et al*., 2003). Moreover, in a recent report concerning a large family of 12 heterozygous carriers of a specific *parkin* mutation (ex3delta40) were asymptomatic (Munhoz *et al*., 2004). In addition, the genotyping of a group of families diagnosed with PD showing anticipation due to late-onset disease in the parent generation and early-onset disease in the offspring, did not support the notion that the presence of single or compound heterozygous *parkin* mutations contribute to this phenomenon (Poorkaj *et al*., 2005). Therefore, at the present moment, although it is certain that heterozygous mutations have an effect on the DA-ergic system, due to heterozygous individuals displaying reduced fluorodopa uptake during positron emission tomography studies (Khan *et al*., 2005), the data remains insufficient to confidently judge the degree to which single hesterozygous *parkin* mutations confer susceptibility to parkinsonism or even typical late-onset PD.

The *parkin* gene encodes for a 465 amino acid protein that contains an N-terminal ubiquitin-like domain, a central linker region and a C-terminal RING domain that consists of two RING finger motifs separated by an in-between RING domain (Thomas & Beal, 2007). It functions as an E3 ubiquitin protein ligase in a similar manner to other RING finger containing proteins by targeting misfolded proteins to the UPS for protein degradation purposes. In addition, it offers

several neuroprotective functions, particularly for ensuring the survival of DA-ergic neurons (Feany & Pallack, 2003). There are studies to suggest that *parkin* mediates neuroprotection by activating IkappaB kinase/nucleur factor-kappaB signaling. This was validated by the finding that *parkin* mutations failed to offer similar protection, by lacking the ability to stimulate the pathway (Henn *et al*., 2007). Furthermore, another study found that the UBL domain of *parkin* interacts with ubiquitin interacting motifs (UIM) of Eps15 (Fallon *et al*., 2006). This adaptor protein is involved in the endocytosis and trafficking of the epidermal growth factor receptor, which, upon interaction with *parkin* it ubiquitinates in a proteasome-independent manner. The gene-protein interaction interferes with EPS15 UIM's ability to bind ubiquitinated EGFR. Subsequently, this step delays EGFR internalization and degradation and also promotes phosphatidylinositol 3-kinase/Akt signaling for cell survival (Fallon *et al*., 2006).

Several *in vivo* studies have indicated a crucial role for *parkin* in modulating mitochondrial functions. This includes a role played in mitochondrial morphogenesis during spermiogenesis (Riparbelli & Callaini, 2007), as well as promoting mitochondrial biogenesis, through the transcription and replication of mtDNA (Kuroda *et al*., 2006). Furthermore, in a fly model of PD, *parkin* seemed to rescue mitochondrial dysfunction, muscle degeneration and DA-ergic cell loss (Yang *et al*., 2006). The mechanism for this was suggested to involve an inactivation of a putative mitochondrial serine/threonine kinase (*PINK1*) that is a further cause of autosomal recessive PD (Clark *et al*., 2006; Park *et al*., 2006). Due to its loss in E3 ligase activity, the mutation manifests as autosomal recessive early-onset PD (Zhang *et al*., 2000; Shimura *et al*., 2000; Kitada *et al*., 1998). The impairment of E3 ligase activity by *parkin*'s post-translational modification involving oxidative or nitrosative stress can also compromise its usual protective function (LaVoie *et al*., 2005). Oxidative damage may also induce solubility alterations in disease-specific mutants of *parkin* as well as in RING-IBR-RING type ubiquitin ligases that show similarness to parkin (Wang *et al*., 2005).

Mutations in the *parkin* gene are a major source of autosomal recessive early onset PD. Cyclin-dependent kinase 5 (Cdk5) interacts and phosphorylates *parkin* at Ser131 of its linker region, thereby blocking autoubiquitylation, thus leading to parkin aggregation both *in vitro* and *in vivo* (Avraham *et al*., 2007). It has been shown that the activation of the Cdk5 pathway presents a particular risk for DA-ergic cell death (Smith *et al*., 2003).

There seems to be an interactive relationship between SNCA and *parkin* that may be particularly prominent to induce alterations of SNCA-mediated mitochondrial functions (Stichel *et al*., 2007).

The claims for a pathological role for mutant *parkin* in PD-related pathoneurogenesis received further validation from evidence gathered from several animal studies. Motor impairments had been observed in flies following the expression of mutant human parkin, while

the insects displayed normal motor function in the presence of wild-type *parkin* (Sang *et al*., 2007). However, this did not produce similar results in an alternative species, since mouse models generated by the targeted deletion of *parkin* failed to produce nigral DA-ergic degeneration. Instead, the study only reported a functional impairment in these mice (Moore *et al*., 2005). This work supports those by others that showed that catecholaminergic neurons from *parkin* knockouts are not more vulnerable towards toxic insults than controls (Thomas *et al*., 2007; Perez *et al*., 2005). Similar findings have been reported for the *SNCA*-induced human disease (Stichel *et al*., 2007; von Coelln *et al*., 2006). These *in vivo* findings suggest that the neuroprotective efficiency of *parkin* seems to be highly selective. Its selective action may depend on the particular neuroprotective pathway involved. It is expected that its identification will assist in elucidating the role that *parkin* deficiency plays in PD pathogenesis.

4.3.5.1.2. PARK6 (PINK1) mutations as a cause of Parkinson's disease

PINK1 [phosphatase and tensin (PTEN) homolog-induced putative kinase 1] is a protein consisting of 581 amino acids that contains an N-terminal mitochondrial targeting sequences as well as a highly conserved protein kinase domain that is similar to the serine/threonine kinases belonging to the Ca^{2+} calmodulin family. Its ubiquitous and punctuated expression pattern suggests for the tendency to localize to mitochondria (Gandhi *et al*., 2006).

Limited information is available on the normal function of the PINK1 gene. However, its mitochondrial localization and the presence of a kinase domain suggest for a role in mitochondrial pathology, protein stability and kinase pathways (Leutenegger *et al*., 2006; Beilina *et al*., 2005). A recent study demonstrated that the human *PINK1* locus is regulated by non-coding naturally occurring antisense RNA, thereby emphasizing its role in mitochondrial biogenesis (Scheele *et al*., 2007). *PINK1*'s mitochondrial-associated physiological function seems essentially to protect. This was demonstrated in several *in vitro* and *in vivo* studies. DA-ergic SH-SY5Y cells show increased vulnerability following exposure to the mitochondrial toxins rotenone and MPPP+ after *PINK1*'s function had been suppressed by siRNA (Deng *et al*., 2005). These results are consistent with another study that showed the overexpression of wild-type *PINK1* capable of preventing starosporine-induced cytochrome c release by the mitochondria with subsequent neuronal apoptosis due to caspase 3 activation (Petit *et al*., 2005).

Mutations present in the *PINK1* gene have been identified as causing early-onset familial PD (Valente *et al*., 2004), although its mutation frequencies vary, particularly among different ethnic groups (Klein *et al*., 2006).

4.3.5.1.3. PARK7 (DJ-1) mutations as a cause of Parkinson's disease

The protein DJ-1 is a highly conserved member of the DJ-1/Thi/PfpI protein superfamily that consists of 189 amino acids. It has ubiquitous expression, localizing to the mitochondria (Zhang *et al*., 2005b). Although extremely rare, accounting for 1-2% of all early-onset PD cases (Hedrich *et al*., 2004b), mutations relating to functional loss in the *DJ-1* locus associate with rare forms of autosomal recessive early-onset parkinsonism (Bonifati *et al*., 2005).

Although its original proposed role was that of an oncogene functioning in sperm maturation and fertilization, it's newly discovered association with pathogenic mutations in familial PD has provided it with a range of newly associated physiological roles. It has been assigned an antioxidant role, due to its inherent ability to self-oxidize, thereby functioning as a scavenger that eliminates the presence of ROS, such as H_2O_2 (Canet-Aviles *et al*., 2004).

Furthermore, the overexpression of wild-type DJ-1 in both a culture-based as well as an *in vivo* model, showed DA-ergic neurons to receive protection against toxin-induced potential oxidative damage (Paterna *et al*., 2007). Its been assigned chaperone activity, after it was observed that DJ-1 displays redox-dependent chaperone function that inhibits SNCA aggregation and cell apoptosis (Zhou *et al*., 2006). Furthemore, the finding that it associates with *parkin* during oxidative stress suggests for a shared role played by both to induce neuroprotection (Moore *et al*., 2005). It has been shown to act as a transcriptional co-activator, believed to result in its antioxidant action. DJ-1 stabilizes the antioxidant transcriptional master regulator nuclear factor erythroid 2-related factor (Nrf2) through a preventative association with Nrf2's inhibitor, Keap1 as well as by ubiquitinating Nrf2 (Clements *et al*., 2006).

PD mouse models that had been induced by knocking-out *DJ-1* were observed to progressively develop age-dependent motor deficits, hypokinesia and DA-ergic dysfunction. However, no nigrostriatal DA-ergic cell loss was observed in either these two studies, raising questions on whether *DJ-1* gene alterations are sufficient to induce full-scale PD (Goldberg *et al*., 2005). However, when challenged with a neurotoxic response evoked by MPTP, the nigrostriatal DA-ergic neurons derived from these *DJ-1* deficient mice revealed increased vulnerability (Kim *et al*., 2005). Although the reason for this is unknown, various mechanisms have been proposed. The reasons proposed include increased p53 and Bax expression (Bretaud *et al*., 2007), deficits in the phase II detoxification enzyme NQO1 (NADPH quinine oxireductase 1) (Clements *et al*., 2006), impaired Na + /K+ ATPase that induces irreversible membrane potential changes (Pisani *et al*., 2006), defective signaling by phosphatidylinositol 3-kinase/*Akt* (Yang *et al*., 2005), and the inability of the apoptotic protein Daxx to inhibit apoptosis signal regulating kinase 1 (ASK1) thereby inducing cell death (Junn *et al*., 2005). Of particular significance to PD also is the finding that *DJ-1* enables the transcriptional upregulation of TH expression (Zhong *et al*., 2006).

Taken together, all the above-mentioned studies on *DJ-1* overwhelmingly suggest for its role in maintaining and mediating the survival of DA-ergic neurons.

4.3.5.2. Autosomal dominant forms of Parkinson's disease

Two genes responsible for autosomal dominant forms of PD have been identified, both appearing to hold major relevance for the typical late-onset sporadic form of the disease.

4.3.5.2.1. PARK1 mutations as a cause of Parkinson's disease

Researchers have identified three missense mutations in the *SNCA* gene, an alanine to threonine conversion at amino acid 53 (A53T), an alanine to proline (A30P) and and glutamic acid to lysine (E46K) (Polymeropoulos *et al*., 1997; Zarranz *et al*., 2004) that, in addition to genomic triplications of a region of the SNCA gene, associates with autosomal dominant PD. Two separate studies have brought the protein SNCA to the centre stage in research concerning PD aetiology. A missense A53T mutation was found in the encoding gene on chromosome 4 in a large Greek family with members that displayed signs of juvenile-onset PD (Polymeropoulos *et al*., 1997). In addition, antibodies used against SNCA stained strongly for LBs and LNs in the PD brain (Spillantini *et al*., 1997). SNCA has an increased propensity to aggregate due to its hydrophobic non-amyloid-β component domain, giving rise to aggregation and abnormal processing.

In a fly (*Drosophila melanogaster*) model of PD, a non-amyloid-β component domain and truncated forms of SNCA mediated neurodegeneration, the formation of large inclusion bodies an increased accumulation of SNCA species of high molecular weight (Periquet *et al*., 2007). β-amyloid seemingly protects against SNCA-induced toxicity. One suggested way in which this may be achieved is by reducing SNCA protein expression (Fan *et al*., 2006), by blocking pore-like oligomers from developing (Tsigelny *et al*., 2007), and by activating *Akt* signaling, thereby promoting cell survival (Hashimoto *et al*., 2004).

Another study provided support to the critical role played by C-terminal truncation as a regulator of SNCA aggregation and DA-ergic toxicity *in vivo* (Tofaris *et al*., 2007). Using mice for expressing C-terminally truncated human SNCA, with a rat TH promoter on a mouse SNCA null background, the animals developed progressive loss of nigral DA-ergic neurons, pathological inclusions and impaired motor function. It was further shown that a pathological modification involving phosphorylation of Ser129 in SNCA promotes its aggregation, and that SNCA phosphorylated by Ser129 forms a major component of LBs (Anderson *et al*., 2006; Smith *et al*., 2005).

Another mechanism that may possibly underlie SNCA aggregation and promote the formation of soluble oligomers is the catalyzation of Ser129 phosphorylation of SNCA by the G-

protein–coupled receptor kinase 5 (Arawaka *et al*., 2006). One of the earliest defects following SNCA accumulation *in vivo* is blockage of the ER to a supply by Golgi vesicular trafficking, thereby disrupting basic cellular functions through increased ER stress (Cooper *et al*., 2006). Furthermore, SNCA has been assigned a crucial role in modulating mitochondrial function during neurodegeneration. Evidence for this derives from transgenic mice expressing the human mutant SNCA (A53T) that developed mitochondrial pathology (Martin *et al*., 2006), providing a crucial role to SNCA in modifying mitochondrial function. The role played by SNCA in mitochondrial pathology has previously been shown, from a study where SNCA depleted mice proved to be resistant to the damaging effects of mitochondrial toxins (Klivenyi *et al*., 2006).

The overexpression of mutant SNCA (A53T & A30P) have also provided valuable clues as to the toxic nature of SNCA. This showed an increase in cytsolic catecholamine concentrations thereby disrupting normal function. In addition, this facilitated the toxicity of oxidized catechol metabolites that have been implicated before to play a role in the selective neuronal degeneration seen in PD (Mosharov *et al*., 2006; Hasegawa *et al*., 2006).

Furthermore, SNCA aggregation has also been assigned a pathophysiological relationship. These include the activation of stress-signaling protein kinase (Klegeris *et al*., 2006), impairing microtubule-dependent trafficking (Lee *et al*., 2006), reducing intercellular communications at gap junctions (Sung *et al*., 2007), and inhibiting histone acetylation in the nucleus, thereby promoting toxicity (Kontopoulos *et al*., 2006).

4.3.5.2.2. PARK8 (LRRK2) mutations as a cause of Parkinson's disease

Point-mutations have been identified in virtually all the known domains of leucine-rich repeat kinase 2 (*LRRK2*). One of them is the G2019S mutation, also known as dardarin (Deng *et al*., 2006), an important cause of autosomal dominant PD (Paisan-Ruiz *et al*., 2004). Increased research focus has been placed on its role in the pathogenesis of PD, due to reports that *LRRK2* mutations may occur beyond mere familial cases. People from certain geographical parts seem especially vulnerable. Most notably are North Africans and those of a Middle Eastern descent, with a frequency rate of 20-40%, while those of European descent are less affected at a prevalence rate of 1-7% (Hardy *et al*., 2006).

Although this protein's precise physiological role is presently unknown, its multiple functional domains suggest for its involvement in various functions. In an attempt to shed light on the functional role it might play, cell-culture and *in vivo* studies were conducted intended to identify the intracellular and tissue-specific location of *LRRK2*, to shed light on its functional role. It was found that the majority of forebrain structures that includes nigrostriatal DA-ergic neurons express *LRRK2*. Moreover, it was found to be predominantly present in the cytoplasm,

particularly in the golgi apparatus, synaptic vesicles, plasma membrane, lysosomes, while it was also found to associate with the mitochondria's outer membrane (Higashi *et al*., 2007; Taymans *et al*., 2006).

Several studies have demonstrated *LRRK2*'s role in synaptic vesicle function. A role for it was assigned in the polarized sorting of synaptic vesicle proteins to axons, with evidence for this derived at by instigating deletion mutants of *LRRK2* homolog in *Caenorhabditis elegans* that depleted synaptic vesicle proteins (Sakaguchi-Nakashima *et al*., 2007). Additional evidence to suggest *LRRK2*'s involvement in synaptic vesicle recycling, neurite outgrowth and functions related to the golgi vesicles, lysosomes and mitochondria have recently been made available. Impairment in any of these aspects may compromise neuronal survival (Li & Beal, 2005). The studies include those that have shown *LRRK2*'s ability to associate with lipid rafts, to localize to LB's as well as to regulate neurite length and branching (Hatano *et al*., 2007; MacLeod *et al*., 2006).

Increased kinase activity in *LRRK2* is believed to be caused by disease-causing mutations of the G2019S and the nearby I2020T that both form part of the N-terminal portion of the activation loop in the kinase domain (West *et al*., 2005; Goeckner *et al*., 2006). This is believed to lead to dardarin's toxic effects (Greggio *et al*., 2006).

Evidence for the association derives from autophosphorylation and the phosphorylation of the generic substance myelin, a basic protein, and comparing this to either the wild-type LRRK2 or to equivalent mutations in paralogous kinase LRRK1 (Greggio *et al*., 2007). Multiple studies have revealed kinase activity in *LRRK2* caused by disease-causing mutations affect cell viability. Disease pathogenesis is believed to result from apoptotic-induced activation of kinase mechanisms (Greggio *et al*., 2006). Although this mechanism is believed to influence signaling, it remains to be ascertained how kinase activity produces this effect. Abnormal protein phosphorylation may be involved, since alterations in the phosphorylation of key proteins have been detected that are involved in MAPK signaling in the leukocytes taken from patients with G2019S mutations (White *et al*., 2007).

4.3.5.2.3. Other genes implicated in Parkinson's disease

Several genes not already mentioned have been implicated in the pathogenesis of PD, or have been re-examined for a possible role to play in the onset of the disease. A meta-analysis done provides support for a role of ubiquitine C-terminal ligase 1 (*UCHL1*) (Maraganore *et al*., 2004). However, the magnitude of the role assigned to the gene has controversial. For instance, controversy surrounds the results of association studies with a polymorphism (S18Y) in the gene, and the cohort on which the suggested role is based on has been criticized, since only a single family, with relatively few members have been described thus far (LeRoy *et al*., 1998).

To date, neither genetic studies nor post-mortem brain studies have been able to inform on the temporal sequence and relationships amongst the various cellular processes. It is hoped *in vivo* animal models can only answer these questions convincingly. However, hitherto, no single toxin or gene-based model has succeeded to fully recapitulate all the pathological and clinical features that define human PD.

4.3.5.3. Relative contribution made by genetic mutations towards the overall incidence of the disease

The aetiology of PD likely involves both environmental and genetic factors that ultimately contribute to the progressive demise of specific neuronal cell populations (Di Monte, 2003). Various studies have demonstrated that the majority of PD cases cannot explained be explained only by simple genetic models. A study performed in Sweden on twins demonstrated a low concordance rate, and the analysis of three clusters of cases found in Canada reported a greater compatibility with an environmental cause, than the contribution of known genes and mutations to the condition. Moreover, a study that examined a large cohort of first-degree relatives of probands (therefore, both population- and referral based) suffering from PD and relatives of control probands, found only a modestly overall increased relative risk. Whereas the relative risk for probands of a younger onset (66 years or younger) revealed a more significantly increased risk, the relatives of probands who manifested symptoms when older had no associated increased risk (Rocca *et al*., 2004). This study confirmed the results of a large population-based study conducted earlier (Marder *et al*., 1996), suggesting that alternative studies that reported disproportionately higher relative risk estimates may have been influenced by referral and ascertainment bias. Recessive inheritance or reduced penetrance may possibly explain the negative family history perceived from studying the profile of previous generations in some studies.

Moreover, a large-scale study designed to asses the relative degree to which genetic versus environmental factors contribute to the aetiology of PD reports a high degree of concordance within twin pairs for early-onset PD (defined as onset before the age of 50). A far lesser concordance was found for disease of late onset (onset >50 years of age) (Tanner *et al*., 1999). The epidemiological findings highlight the genetic causes that might be responsible for the majority of early-onset PD cases, whereas onset after a patient's 50^{th} birth-year may possibly increase due to the impact of environmental factors instead. However, the etiologic role played by a shared environment can never be completely excluded.

4.3.6. Drug-induced Parkinson's disease

A number of pharmacological agents for lesioning specific neuronal populations are used for mimicking the pathological and functional alterations that characterize a disorder. These models are useful for evaluating therapeutic strategies for symptomatic relief and are also used to test the neuroprotective capacity of substances. In the current context, the capacity of certain substances to disrupt or destroy the catecholaminergic systems has been used extensively to induce a selective loss of midbrain DA-ergic neurons and develop PD models in different animal species, mainly rodents and non-human primates. However, on the basis of experimental and clinical findings, PD was the first neurodegenerative disease to be modelled in animals.

An association between drinking well water, rural living, and exposure to chemicals commonly used for agricultural purposes, with increased risk for PD developing has been reported (Liou *et al*., 1997; Priyadarshi *et al*., 2001). Further epidemiological evidence to support an association between past occupational exposure to pesticides and increased risk of developing AD or PD (Baldi *et al*., 2003), while an inverse, apparently protective relationships between cigarette smoking, coffee consumption and PD has also been reported (Hernan *et al*., 2002). The ability of certain common environmental agents, such as pesticides, to accurately reproduce the key pathological features of the disease has led to the widely-supported postulation that exposure neurotoxic agents may increase the risk for developing PD (Gorell *et al*., 1998; Menegon *et al*., 1998). This will be further discussed in the section to follow.

Chemical agents that block the DA D_2 receptors located in the striatum count amongst the most frequent causes of a condition clinically referred to as drug-induced parkinsonism (DIP). This includes antipsychotics, anti-emetics, as well as calcium antagonists. DIP occurs regularly, with figures of 4-37% of all patients quoted (Gershanik, 1994; Bower *et al*., 1999).

DIP was first recognised as a clinical syndrome shortly after the introduction of the first antipsychotics in the 1950's. Subsequent investigation following the clinical observation that their use often associate with acute or tardive dyskinesia, it was found that they block striatal DA D_2 receptors, to inhibit them from performing their usual movement-related function. Whereas the "classic" antipsychotics work by blocking D_2 receptors, thereby producing catalepsy and terminating amphetamine-induced stereotypical behaviour, more recently developed ones, such as clozapine, risperidone and quetiapine are not associated with these effects. Moreover, these modern "atypical" antipsychotics cause less than 40-70% of D_2 occupancy, which is probably too low to produce parkinsonism (Hirose, 2006), while it is also believed that the binding of these drugs to D_2 receptors dissociate effortlessly. Therefore, the D_2 receptors remain accessible to intact DA transmission (Seeman, 2002).

Anticholinergic compounds are used for treating rigidity and tremor associated with PD, and therefore, cholinergic drugs, such as *cisapride* may also provoke or worsen tremor (Sempere *et al*., 1995). In addition, a range of commonly-prescibed medicines are reported to occasionally

induce parkinsonism. Although a clear relationship between the compound and the emergence of PD remains to be established, the reports do raise concerns, while an investigation as to the validity of these claims should be sought. Possible disease-aggregating compounds include *amiodarone*, a cardiac antiarrhythmic drug that possibly provokes thyrotoxicosis that could induce the onset of tremor (Armon, 2007; Dotti & Federico, 1995). In addition, *lithium* often associates with postural tremor (Ghadirian *et al*., 1996), while a range of selective serotonin reuptake inhibitors is known to induce DIP, or to at least increase pre-existing PD in approximately 10-15% of patients (Gerber & Lynd, 1998; Richard *et al*., 1999). Furthermore, in patients with an underlying disturbed blood barrier barrier (BBB), the antifungal drug *amphotericin B* may produce leukoencephalopathy to give rise to reversible parkinsonism (Walker & Rosenblum, 1992; Balmaceda *et al*., 1994; Mott *et al*., 1995).

A drug commonly used for treating alcoholism, *disulfiram*, metabolises to carbon disulfide, and has neurotoxic properties and can also induce lesions in the pallidum and SN (Richter, 1945), with co-occurance of parkinsonism (Laplane *et al*., 1992; de Mari *et al*., 1993). It is also worth the mention that several reports describe an association between the use of the chemotherapeutics *cyclosporine cytosine arabinoside* and *cyclophosphamide* and DIP. This particularly seems to be the case in patients who have undergone bone marrow or organ transplantations (Mott *et al*., 1995; Wasserstein & Honig, 1996).

Various *in vitro* studies have raised the possibility that the DA-agonist L-DOPA may be toxic to DA neurons. However, no study has been able to confirm these speculations in a physiologically relevant animal model. Although long-term L-DOPA use is associated with the onset of debilitating motor complications, these are believed to be a reversible adverse effect, rather than the result of a toxic insult on the nerve cells. This matter will be returned to at a later stage in the current chapter.

For approximately 60% of all patients, DIP typically manifests within 1 month of starting the offending drug regimen, while they usually appear within 3 months in the remaining cases. The general risk for developing DIP is estimated to be between 10-40% (Caligiuri *et al*., 1999; Kapur *et al*., 2000;). The main risk factors for developing DIP are identified to be the dose and potency of the drug take, advanced age, female gender, the presence of pre-existing subclinical depletion of DA or parkinsonism (Caligiuri *et al*., 1999; Llau *et al*., 1994; Marti Masso *et al*., 1996).

The main diagnostic criterion for DIP is therefore the disappearance of symptoms after a delay of up to a year following withdrawal of the drug, to suggest underlying idiopathic PD or another related nigrostriatal disease. Therefore, DIP is believed to be reversible. However, full recovery may take up to 6 months (Marti Masso & Poza, 1996), while one study suggests that DIP may persist for up to 2 years subsequent to the withdrawal of the offending drug (Garcia-

Ruiz *et al*., 1992). The time-course necessary for recovery may result from the type of drug causing the effect, with antipsychotics generally inducing parkinsonism within a 6 month period after first taking the drug, while calcium channel blockers, such as the piperazine derivatives *flunarizine* or *cinnirazine*, generally take longer (9-12 months) for parkinsonian-like symptoms to subside (Llau *et al*., 1994).

The initial clue to achieving a differential diagnosis of PD should be to establish whether the patient has a history of using drugs known to evoke DIP. Ascertaining a history of toxin exposure proves more troublesome, since patients are often not aware of the toxic properties of compounds. An additional line of investigation concerns the clinical syndrome the patient presents with, with DIP and toxic parkinsonism patients that often present with atypical parkinsonism with non-parkinsonian signs and symptoms. Moreover, most of these patients show no sign of benefiting from DA-ergic agonist treatment. Since DIP and toxin-evoked PD often damage the pathway at a downstream location that is involved in producing movements, or block postsynaptic D_2 receptors, L-dopa or other DA agonists prove to be of no use, since the D_2 receptors that they usually act on are blocked by the antipsychotic medication. Therefore, imaging techniques such as PET and SPECT may be useful to distinguish sporadic-onset PD from DIP or toxin-induced PD, since they often reveal that PD is a disease that affects the presynaptic DA receptor, whereas DIP and toxin-induced forms predominantly cause postsynaptic degeneration.

4.3.7. Toxin-induced Parkinson's disease

Medical discoveries typically start with clinical observation, eventually proceeding towards attempts to artificially induce clinical conditions in the laboratory (Peller, 1967). PD is regarded as a complex entity that most probably manifest due to a combination of environmental triggers, an underlying genetic susceptibility and the natural decline seen during old age.

It has been suggested that exposure to environmental toxins, such as pesticides and herbicides, either on their own or in synergy with endotoxic and/or genetic predisposing factors, may contribute towards damage sustained by DA neurons (Van den Eeden *et al*., 2003), especially when exposure takes place during the developmental period, when neurons are particularly vulnerable. The four most popular agents used for recapitulating PD are MPP^+, the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), the noradrenergic analog 6-hydroxydopamine (6-OHDA), rotenone (RT) and paraquat, a compound that is structurally similar to MPP^+. All of these are mammalian models capable of evaluating therapies transferable to the clinic, and testing in non-human primate models is often the first initial step before a clinical trial can commence (STAIR, 1999). All of these cause extensive degeneration of nigrostriatal DA neurons and mitochondrial dysfunction, with an unbiased count of surviving

DA-ergic neurons in the brains of toxin-exposed animals that remains the ultimate validation of toxin-induced neuronal destruction (Emborg, 2004). Since pesticide exposure in animals simulates the characteristics associated with the PD phenotype (Menegon *et al*., 1998; Gorell *et al*., 1998), a leap in understanding was made that by exposing animals to similar agents, knowledge concerning possible causes of the disease could be expanded on.

An accurate *in vivo* experimental model of PD should simulate the core pathology seen in human PD in animals also, such as a progressive, selective nigrostriatal DA-ergic degeneration and LB formation, as seen in post-mortem PD brains. In addition, it must be able to effectively assess the relevance of the systemic oxidative damage and mitochondrial impairment in the pathogenesis of PD, and explain the potential role played by pesticide exposure in the development of Parkinsonism. Currently, no existing animal model incorporates all the complex chronic neurodegenerative features associated with the disease. However, specific chemical compounds are used with the aim of disrupting the nigrostriatal DA-ergic pathway and to mimic the striatal DA deficiency seen in PD patients. In addition to this central hallmark, the various agents provide additional advantages and disadvantages over each other.

4.3.7.1. Reserpine-induced parkinsonism

Although reserpine (RES)-use is far less commonly found in the methods section of current animal-related research done on PD nowadays, its use has allowed for a major shift in our current understanding of one major patho-mechanism underlying the disease. Carlsson *et al.* (1957) demonstrated in rabbits that the drug evokes catecholamine depletion in the brains of experimental animals, observed as akinetic behaviour, subsequently relieved by the systemic administration of L-DOPA. Moreover, several other clinically used antiparkinsonian drugs (e.g., DA agonists, L-DOPA+benzerazide, amantadine and trihexyphenidyl) were shown to improve RES-induced akinesia, proving that the behavioral recovery was DA-dependent (Menzaghi *et al*., 1997). After this radical postulation was also confirmed in a human clinical population (Hornykiewicz, 1963), the most common hypothesis concerning PD etiology was established, that PD's motor symptoms result from striatal DA deficiency (Bernheimer *et al*., 1973). RES-induced hypokinesia in rodents were revealed to be due to lost storage capacity of the intracellular vesicles of monoamines (Hornykiewicz, 1966), through magnesium- and ATP-dependent mechanisms (Carlsson, 1975).

RES was commonly in use as a centrally acting hypertensive drug, up until Carlsson's finding that it also acts as a presynaptic DA depleting drug, when it was withdrawn from clinical use. As experience with RES's and knowledge concerning its workings increased, investigators expressed their concern as to the suitableness of the model relative to its clinical phenotype.

However, despite the concern for RES-induced changes, these were found to be only temporary and the direct administration of RES into the striatum of animals failed to produce the morphological changes associated with the disease that are expected to be present in the DA-ergic neurons of the SN (Betarbet *et al*., 2002). Subsequently, these nonspecific effects by RES on all variants of monoaminergic neurotransmission (Gerlach & Riederer, 1996) evoked enough concern for the model to be considered inadequate to fit its purpose, especially when compared to other animal models (Gerlach & Riederer, 1996). In addition, it was found to stimulate the release of neurotransmitters that are not thought to be directly involved with the clinical condition. However, some studies suggest that other neurotransmission pathways (e.g., serotonin and noradrenaline) may be involved in the association between PD and the onset of depressive symptoms (Barbosa *et al*., 1997).

4.3.7.2. MPTP-induced parkinsonism

The systemic administration of the designer drug 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) accurately produces a Parkinson-like syndrome in both human and nonhuman primates (Langston *et al*., 1983) and provides the most effective experimental model of PD (Jenner, 2003). MPTP is metabolized to 1-methyl-4-phenylpyridinium (MPP+) by monoamine oxidase B present within glia (Chiva *et al*., 1984), whereupon it is taken up into DA-ergic neurons by means of a dopamine reuptake pump (Jatvich *et al*., 1985). Meperidine is an analogue of MPTP that is well-known to produce a severely debilitating form of PD (Langston *et al*., 1983). However, the symptoms of meperidine-induced PD have been shown to respond favourably to treatment (Lieberman & Goldstein, 1985).

MPTP-analogues continue to be used for agricultural purposes. This provokes concerns, in light of the epidemiologically confirmed relationship between chronic exposure to herbicides and PD developing (Liou *et al*., 1997; Dinis-Oliveira *et al*., 2006; Brown *et al*., 2006).

4.3.7.3. Rotenone-induced parkinsonism

Chronic systemic exposure to the lipophilic pesticide rotenone reproduces the anatomical, neurochemical, behavioral and neuropathological features of PD. These include selective nigrostriatal DA-ergic degeneration and generating cytoplasmic inclusions containing SNCA and ubiquitin (Betarbet *et al*., 2002; Sherer *et al*., 2003). Rotenone has two intracellular molecular targets, namely inhibiting complex I of the mitochondrial electron transport chain (Chance *et al*., 1963), as well as to evoke a microtubule-depolarizing action (Brinkley *et al*., 1974; Marshall and Himes, 1978). It shares these characteristics with that of MPP^+, the active metabolite of the experimental PD toxin MPTP, which also inhibits complex I (Dauer & Przedborski, 2003) and depolymerizes microtubules (Cappeletti *et al*., 1999; Cappeletti *et al*., 2001). It is yet to be

determined whether paraquat, a structural analogue to MPP^+ (Di Monte, 2003) delivers a similar microtubule depolarizing effect for cell-selective toxicity, as other environmental toxins linked to PD does. Neither of these effects, however, explains the selective vulnerability of DA-ergic neurons to rotenone satisfactorily, since neither are cell-type specific. However, it has been shown that catecholaminergic neurons are rendered particularly vulnerable to this environmental PD toxin when these known activities are combined, since these neurons depend on long-range microtubule-based transport for moving its oxidized cargo. Since rotenone disrupts microtubule-based transport of DA vesicles, DA accumulates in the soma, resulting in increased oxidative stress due to the oxidation of DA, which has leaked from the vesicles (Eisenhofer *et al*., 2004). Moreover, it has been shown that neurons that synthesize neurotransmitters that are not susceptible to oxidative stress, such as glutamatergic or GABAergic neurons, are more resistant to rotenone toxicity (Ren *et al*., 2005).

In contrast to the rotenone-model (discussed in the section below), intraneuronal inclusions reminiscent of early LBs have occasionally been described in the MPTP animal model (Kowall *et al*., 2000), a mitochondrial complex I inhibitor, that was discovered when humans were accidentally exposed to its toxic metabolite, MPP^+ (Langston *et al*., 1983). Yet the presence of LBs in the cytoplasm is not detected consistently, and the ones reported in MPTP-treated baboons differ from those found in human brains (Kowall *et al*., 2000). Moreover, an absence of LBs were found in post-mortem human brains, that had developed parkinsonism due to the toxic effects of intravenous MPTP administration (Langston *et al*., 1999), to suggest that the neuropathology may result from factors that are additional to a single toxic exposure. Due to the lipophilic nature of the compound, MPTP easily crosses the BBB, thereby allowing for systemic (Burns *et al*., 1985; Langston *et al*., 1984) or intracarotid infusion (Bankiewicz *et al*., 1986). Combinations of systemic and intracarotid administration have also been performed, in order to influence the pathological consequences and speed of syndrome onset (Oiwa *et al*., 2003). Mice and monkeys are better candidates for the MPTP-model, in contrast to rats that display increased resistance to the effects of the toxin (Giovanni *et al*., 1994a; Giovanni *et al*., 1994b).

4.3.7.4. 6-OHDA-induced parkinsonism

The catecholaminergic neurotoxin 6-OHDA was the first chemical agent discovered to reveal preferential toxic effects on the catecholaminergic pathways (Ungerstedt, 1968) of both the peripheral as well as the CNS. Since it is unable to cross the blood brain barrier, the drug is injected intracerebrally, by stereotactic lesioning of the nigrostriatal pathway, the SN or the striatum. It exhibits a high affinity for various catecholaminregic plasma membrane transporters, including for dopamine (DAT) and noradrenaline, to allow it to concentrate in both DA and noradrenergic nerve terminals. The 6-OHDA mode of action involves the combined effect of

ROS and quinones that stems from its ability to oxidize to superoxide species, such as H_2O_2, and *para*-quinone (Saner & Thoenen, 1971; Heikkila & Cohen, 1971). Once it accumulates in the brain parenchyma, it rapidly degenerates the DA-ergic neurons (within 24 hours), while striatal DA diminishes 2 to 3 days later (Zigmond & Keefe, 1997). The toxin therefore leads to large-scale destruction of nigrostriatal DA-ergic neurons. Although this makes the model valuable for assessing gross motor impairments, this aspect limits its use for exploring more subtle motor deficits.

Systematic intracerebral injections of 6-OHDA led to the first stereotaxic mapping of the monoamine pathways present in the rat brain to be proposed (Ungerstedt, 1971), as well as an analysis of rotational behaviour, as a classical behavioral test useful for screening drugs with potential therapeutic application in treating a range of neuropathologies.

The 6-OHDA experimental parkinsonian model has been produced in many species, including mice, rats, cats, dogs and non-human primates (Zigmond & Stricker, 1989). Major differences in terms of magnitude of the lesion have been detected based on the amount of 6-OHDA administered, the anatomical site of the injection and inherent differences between animal species as to sensitivity shown towards the substance (Betarbet *et al*., 2002), thereby preventing the method from currently being standardized in any way. However, rats remain the most commonly used species due to its high degree of replicability and stability, with only minor degrees of individual variance (Emborg, 2004). The model has been criticized for inaccurately simulating all the clinical and pathological features that characterize PD, such as a failure to produce an effect on the locus coerulleus or the lack of typical LB-like inclusions in mammalian-models. However, despite these limitations, the model provides an efficient route by which to ascertain the efficacy of compound that hold promise for use in parkinsonian-related activities, such as for determining the value of cell transplants and neurotrophic factors (Dunnett *et al*., 1983). A striatal injection of 6-OHDA induces a retrograde degeneration of the mesostriatal dopamine pathways (Rodrigues *et al*., 2004). However, in PD, as opposed to experimental PD, neurons may also degenerate in an anti-retrograde manner.

Due mainly to practical considerations, 6-OHDA has been employed mainly in small animals such as rodents, although attempts have been made to administer the toxin to non-human primates as well (Crofts *et al*. 2001).

4.3.7.5. Metamphetamine-induced parkinsonism

Metamphetamine is used for the drug's ability to stimulate DA release (McMillen, 1983), resulting in DA depletion at the level of DA-ergic nerve terminals, albeit showing minimal reactions within the nigral cell bodies (Fibiger & Mogeer, 1971). Yet, the mechanism with which metamphetamine achieves its effects remains debatable. However, the strongest evidence thus

far suggests that metamphetamine exerts its neurotoxic effects *via* the DA receptor and the DA transporter (DAT). These studies, that showed how selective antagonists are able to block its toxic mechanism of action (Sonsalla *et al*., 1986; Sonsalla *et al*., 1989), remains, however, an indirect means by which to derive at a conclusion. An alternative explanation suggests that a disturbance in the cellular energy metabolism, specifically oxidative stress, due to DA autoxidizing and excitotoxicity (Kostrzewa et al., 2002) may be responsible for the adverse effects exerted on neurons. The ideal candidates for metamphetamine administration are rodents and non-human primates that incur neurotoxic effects when the drug is administered at very high doses (Wagner *et al*., 1980; Wagner *et al*., 1979). However, major concern with regard to the applicability of the disease towards the clinical phenotype, is that histochemical profile associated with PD, namely degenerated DA-ergic nerve terminals and intracellular inclusions have not been detected. Further criticism is that it is useful as an acute model of striatal DA depletion only, and that PD follows fundamentally a chronic, progressive course. Yet, despite these limitations, metamphetamine continues to be used extensively in biochemical and physiological studies that focuses on the effects of striatal DA depletion.

4.3.7.6. Paraquat-induced parkinsonism

Systemic exposure of rodents to the herbicide paraquat, alone or in combination with the fungicide, maneb, accurately reproduces certain pathological features of PD. This includes the formation of intraneuronal deposits of SNCA and the selective degeneration of DA-ergic neurons in the SN (Manning-Boð *et al*., 2002; McCormack *et al*., 2002; Thiruchelvam *et al*., 2003; Peng *et al*., 2004). Chronic treatment with paraquat or paraquat/maneb was also shown to activate microglia. Although the relevance of paraquat mediated glial activation remains to be demonstrated (McCormack *et al*., 2002; Saint-Pierre *et al*., 2006), so far epidemiological studies have shown a lower risk for PD associated with regular use of non-steroidal anti-inflammatory drugs (Chen *et al*., 2005). In addition, an abundance of reactive microglia, as mediators of neuroinflammation were found in post-mortem SN, particularly around nerve cells (Langston *et al*., 1999). As was explained in section 4.3.4. of the current chapter, a so-called “priming” effect may possibly be responsible for neuronal cell death that stems from exposure to paraquat.

Since PD is likely to have a multifactorial etiology, insight as to the mechanisms that underlie toxin-mediated cell death, could help to identify conditions that enhance the vulnerability of DA-ergic cells to degenerative processes. Since this “priming effect” of paraquat may be the toxic consequences of NADPH-oxidase-catalyzed reactions such as the ability to redox cycle, which a variety of other naturally occurring and synthetic molecules also show the ability for (Frank *et al*., 1987), a range of toxic agents may be particularly relevant to neurodegenerative processes that selectively target DA-ergic neurons.

4.3.8. Parkinson's disease induced by sustaining mechanical trauma

Mechanical brain damage resulting from traumatic head injury can result in the same core symptoms by which PD is recognised. Epidemiological data also suggest for an association between the incidental rate of sporadic neurodegenerative diseases and a history of mechanical stress, such as head injury. On average, it takes 30 years from when the injury occurred, for the first PD symptoms to appear (Taylor *et al*., 1999; Bower *et al*., 2003; Semchuk *et al*., 1993). In addition, LB formation (and SNCA aggregation) has been found in the brains of trauma victims similar to the brains of PD patients (Uitti & Calne, 1993; Uryu *et al*., 2003).

5. Differentially diagnosing Parkinson's disease

Whereas the cause of primary parkinsonism that makes up the majority of PD cases, are sporadic and of unknown aetiology, causal agents have been identified for certain forms of the disorder. These are also referred to as secondary parkinsonism. While the motor-related symptoms were initially considered to be unique to PD, it later became appreciated that other neurodegenerative disorders, including multiple system atrophy and progressive supranuclear palsy also manifest some or all of these clinical features. Other conditions include certain infections (encephalitis lethargica), intoxications (manganese, carbon monoxide poisoning and methylphenyltetrahydropyridine), iatrogenic conditions (DA-ergic antagonists) and certain disorders of the CNS (i.e. hydrocephalus and infarctions) equally exhibit some of these symptoms. In light of the awareness that various clinical conditions can co-occur with some of the core symptoms of parkinsonism, it was necessary to develop a system that will allow for differentiating idiopathic PD from other related conditions.

Idiopathic PD is a generic term used for describing the movement disorders that are caused by the degeneration, infection, injury, or intoxification of the DA-ergic neuronal system, which compiles the most common cause of parkinsonism (Yahr, 1982). Although PD is hallmarked by an imbalance between DA-ergic inhibition and cholinergic excitation via the depleted state of endogenous DA levels, numerous other defective mechanisms may underlie the eventual manifestation of the disease. Although the term classically means "to have no known cause", this term is not strictly true in light of the plethora of newly discovered genetic mutations that were discussed in section 4.3.5.

Although the diagnosis of PD may conceal a far-ranging pattern of related disorders that differ only in terms of its aetiology and course, and therefore challenge clinicians to distinguish idiopathic PD from other clinical phenotypes. Classification schemes with limited criteria that need to be met have been developed. There are currently no clinical tests available with which to confirm a diagnosis of idiopathic PD. However, a positive response to the synthetic

administration of dopamine (levodopa/L-DOPA), a DA precursor that provides symptomatic relief from symptoms due to the disease's inherent DA deficiency, separates idiopathic PD from disorders that may present a similar clinical profile.

Neurological diseases that are characterized by the presence of involuntary hypokinesia, hyperkinesias, or the abnormal execution of movements, in the presence of definite consciousness, are classified as movement disorders, also sometimes referred to as dyskinesias. Dyskinetic disorders refer to functional anomalies that are due to a disruption of the DA-modulated, cortico-striato-pallido-thalamocortical neuronal circuitry (the so-called extrapiramidal system). This definition, based on the patho-origins of the disease, exclude certain forms of movement disorders from the mainstay definition of dyskinetic disorders. Examples of these are epileptic fits that occur in the presence of impaired consciousness, as well as motor dysfunction induced by upper and/or lower motor neuron diseases that has a purely muscular profile. Moreover, arthrogenic disorders are also not defined as dyskinetic disorders.

To achieve a differential diagnosis for PD often proves an exceedingly difficult task. However, certain clinical tools may be useful, and could possibly be underutilised in clinical practice. Olfactory testing could be a useful adjunct to clinically distinguish between idiopathic PD and related conditions, i.e. vascular parkinsonism (Katzenschlager *et al*., 2004), dementia with LBs (Liberini *et al*., 1999). Although all these pathologies clinically present with disturbed olfactory function, the degree and stage of onset during disease progression differs. In addition, olfactory function is only mildly impaired in multiple system atrophy (Wenning *et al*., 1995), while appearing normal in progressive supranuclear palsy (Wenning *et al*., 1995), corticobasal degeneration (Wenning *et al*., 1995) and parkin-positive parkinsonism (Khan *et al*., 2004).

6. Therapeutic strategies for treating Parkinson's disease

6.1. Pharmacological treatment of Parkinson's disease

Although a number of decades have elapsed since PD was first recognized as a disease entity in the early part of the 19th century, no treatment to either induce a permanent cure or that prevents the progressive pathology exists. The reason for this is that the exact mechanisms that underlie the neurodegeneration continue to remain elusive.

Standard therapeutic guidelines for PD entail administration of DA agonists as well as other DA-ergic analogues (Hingtgen & Siemers, 1998). The therapeutic aim of these is to correct unbalanced striatal DA-levels that form a prominent characteristic of PD.

In 1967, George Cotzias and colleagues (Kordower & Goetz, 1999) were the first to introduce L-DOPA (Levodopa, LARODOPA, DOPAR, L-3,4-dihydroxyphenylalanine), an orally

administered DA-ergic precursor (Fig. 4). L-DOPA therapy can have dramatic effects on PD symptoms, by near complete improvement of tremor, rigidity, and bradykinesia.

The introduction of L-DOPA into clinical practice brought about a revolution in treating the disease, and it soon became regarded as excellent pharmacotherapy for what was previously seen as essentially an untreatable disorder. Its acceptance as the standard therapeutic regime for treating the disease dramatically reduced the need for invasive surgical treatment, which was the sole treatment option available at that stage. The drug remains the most common agent in PD treatment (Hardman & Limbird, 2001).

After its oral administration, the drug is rapidly taken up from the small bowel through active transport mechanisms specific for aromatic amino acids. Plasma levels peak between 0.5 and 2 hours following oral ingestion, and lasts 1-3 hours. The intake of L-DOPA with food may delay its absorption and reduce its peak plasma concentrations, due to dietary amino acids competing for absorption sites in the small bowel. The drug crosses the BBB by a carrier of aromatic amino acids, and comprises an active process. Competition between dietary protein and the drug may again impair L-DOPA's absorption at this level.

In the brain, L-DOPA exerts its effects by converting to DA *via* decarboxylation. This alteration primarily takes place within the presynaptic terminals of DA-ergic neurons in the striatum. After release, DA is either transported back into DA-ergic neurons by the presynaptic uptake mechanism, or metabolised by monoamine oxidase (MAO) or catechol-*o*-methyl-transferase (COMT) (Mouradian & Chase, 1994).

L-DOPA, along with other DA-like agents, such as Carbidopa, Bromocriptine, Deprenyl and Amantadine is often described as the "gold standard" of medicinal therapy currently available to PD sufferers (Kordower & Goetz, 1999). L-DOPA's co-administration with carbidopa, a peripherally acting inhibitor of aromatic L-amino acid decarboxylase is common practice. This therapy prevents L-DOPA's decarboxylation by enzymes in the intestinal mucosa and other peripheral sites. This combination therapy lets a greater fraction of DA enter the CNS, thereby enhancing its central effects.

However, the therapy associates with the onset of severe drug-induced complications, which restricts its practical use (Jankovic, 2006). Both the therapeutic efficiency as well as the adverse effects of L-DOPA are due to the decarboxylation of L-DOPA to DA. These manifest mainly as motor fluctuations, particularly as disabling dyskinesias (Calon *et al*., 2003). Early on during disease pathogenesis, a single dose of L-DOPA provides long-lasting symptomatic relief, often in excess of 4 hours, despite the drug's inherently short plasma half-life (Muenter & Tyce, 1971). However, due to a combination of sustained drug-treatment and the progressive pathology of the disease, the period that the patient stands to benefit from treatment following a single dose of L-DOPA shortens progressively. This 'wearing-off' effect eventually becomes

approximately equal to the plasma half-life of the drug (Nutt & Holford, 1996; Nutt, 1987). To manage this requires ever-increasing amounts of the drug at greater frequencies in order to achieve consistent therapeutic results (Kordower & Goetz, 1999; Lozano *et al*., 2000). Subsequently, increasingly severe motor complications or excessive and abnormal involuntary movements (dyskinesia) manifest with ever increasing drug-doses (Schrag & Quinn, 2000). In addition, the results from the highly anticipated Earlier vs. Later Levodopa (ELLDOPA) clinical trial, also suggest that motor complications are dose-related (Fahn *et al*., 1999).

The fluctuations in L-DOPA's plasma-levels directly translate to fluctuations in striatal DA levels, resulting in pulse-like delivery of L-DOPA, instead of a continuous flow. This could activate post-synaptic signal transduction mechanisms, leading to altered neuronal firing patterns in the pathway that contains the GPi, STN and the GPe-STN-GPi structures. Consequently, treatment regimens that make use of standard formulations that consist of short-acting L-DOPA result in marked oscillations in both synaptic and striatal DA levels (Abercrombie *et al*., 1990; Miller & Abercrombie, 1999; de la Fuente-Fernandez *et al*., 2004; Tedroff *et al*., 1996). Therefore, the exposure of striatal DA receptors to either extremely high or very low levels of DA, instead of a constant concentration, results in intermittent, discontinuous stimulation of the DA receptors. This effect, also known as 'pulsatile stimulation', destabilizes the DA-denervated basal ganglia.

Furthermore, patho-physiological pulsatile stimulation results in a range of expressional changes within the striatal neurons at the gene and protein-level, including preproenkephalin, preprodynorphin, ∆fos-B and DA-receptor-related signals (Calon *et al*., 1998; Cenci *et al*., 1999; Aubert *et al*., 2005; Papa *et al*., 1991; Filion *et al*., 1991; Heimer *et al*., 2002; Picconi *et al*., 2003). As the disease progresses further, the ability of patients to respond to individual doses of L-DOPA may completely disappear ('no-on') and unpredictable fluctuations between 'on' and 'off' periods, often referred to as the 'on-off' or the 'yo-yo' phenomenon is observed (Marsden & Parkes, 1976). Therefore, in relation to time, a marked change in the patient's pattern of response to L-DOPA therapy is observed. These have been attributed to a central pharmacodynamic effect, since they don't associate with any pharmacokinetic L-DOPA changes and are evoked by the DA-ergic agonist apomorphine, which is not stored in the striatal nerve terminals (Fabbrini *et al*., 1988). These alterations potentially form the molecular basis that underlies L-DOPA induced dyskinesias and attempts are made to correct these by implanting novel delivery systems into the brains of PD victims, allowing for continuous L-DOPA delivery.

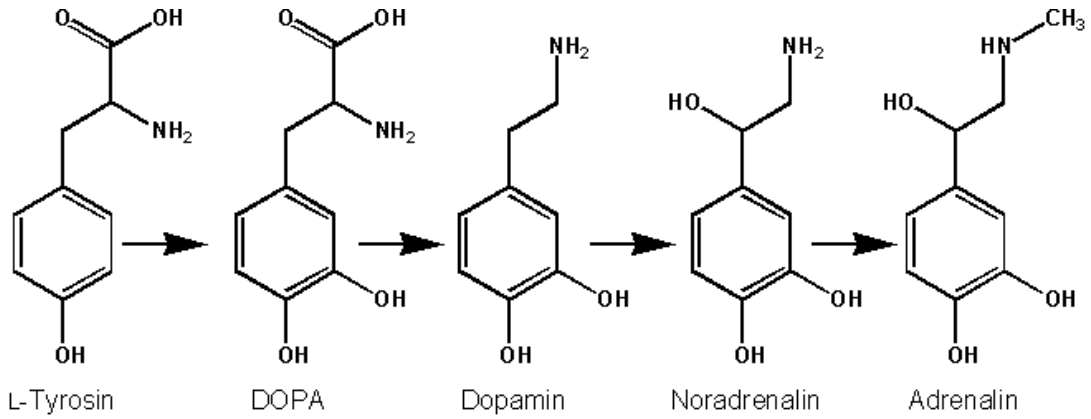

Fig. 4. A schematic diagram illustrating how L-DOPA is converted from Tyrosine hydroxylase to dopamine, noradrenalin, and eventually adrenalin.

The effective treatment of L-DOPA motor and non-motor complications present one of the most challenging domains of PD research (Jankovic, 2005). However, the use of L-DOPA is limited, due to the onset of debilitating motor complications that is particularly troublesome in patients who display symptoms at a young age (Jankovic, 2006). Since these relate to the relatively short half-life that is inherent to L-DOPA's, it has been suggested that DA replacement therapies induce discontinuous or pulsatile non-physiological stimulation of DA receptors located in the striatum, with the initial "bursting" effect that soon surpasses (Olanow & Obeso, 2000; Obeso *et al*., 1994). Continuous stimulation of striatal DA-ergic receptors with DA-ergic agonists has been proposed as an alternative therapeutic strategy for the management of these (Chase *et al*., 1989; Nutt *et al*., 2000; Olanow *et al*., 2000). The rationale for this therapy is that, under normal conditions, DA-ergic neurons in the basal ganglia fire in a random, yet continuous manner, thereby maintaining constant DA concentrations. However, these levels are substantially depleted in the parkinsonian brain, and therefore intermittent oral doses of L-DOPA induce discontinuous, pulsatile, wave-like stimulation of striatal DA receptors, with concomitant molecular and physiological changes that occur in the basal ganglia. Therefore, it is hoped that this novel form of therapy could delay or even altogether prevent the onset of debilitating dyskinesias. A number of clinical studies have also demonstrated how continuous subcutaneous delivery of a DA agonist (e.g. lisuride or apomorphine), or the intrastriatal infusion of L-DOPA can reduce both motor fluctuations and dyskinesia in L-DOPA treated patients suffering from PD (Katzenschlager *et al*., 2005; Manson *et al*., 2002).

Another aspect of L-DOPA treatment that causes concern is that the production of free radicals from the metabolism of DA may contribute to nigrostratal cell death. Therefore, the addition of L-DOPA may actually accelerate the process (Olanow *et al*., 2000). In addition,

another common side-effect associating with the use of this medication is the induction of hallucinations and a confused-state. These are particularly severe in the elderly and those suffering from previous cognitive disabilities. Treatment with traditional anti-psychotics, such as phenothiazines can worsen parkinsonian symptoms, probably through actions at the D_2 receptor level. A recent approach is to use atypical antipsychotics (e.g. clozapine and quetiapine) instead. These are effective at treating psychosis, but without the adverse motor effects associating with the use of typical antipsychotics that may worsen parkinsonism (Friedman & Factor, 2000). Other side-effects of L-DOPA include orthostatic hypotension, cardiac arrhythmias, while its abrupt withdrawal may precipitate neurologic malignant syndrome (Hardman & Limbird, 2001).

Despite its adverse reactions, L-DOPA remains the most common agent for treating the disease. Yet the need for better and more physiologically based types of therapy for treating this complex condition has been recognised. Subsequent to this realisation, a number of novel therapeutic options have been proposed and are currently being developed as either an alternative to L-DOPA therapy or to use in combination with PD's standard therapeutic regimes. However, given the clinical benefits provided to virtually all patients with PD through the use of L-DOPA, all new agents are measured against the 'gold standard' for PD therapy. One drug class that has been earmarked for it's possible complimentary benefits to the PD therapeutic regime, is the orally administered 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, also known as statins (see Fig. 5 for the chemical structure of the statin, simvastatin). These agents inhibit the rate-limiting enzyme HMG-CoA reductase that catalyzes the reduction of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonate during the synthesis of cholesterol (Rossi, 2006).

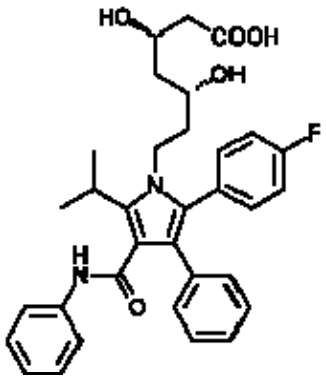

Fig. 5. Chemical structure of the drug simvastatin®.

However, despite the promise of additional therapeutic benefits associated with these drugs, research into their mechanism of action has been limited. This aspect of the study is elaborated on in chapter 7.

Another standard therapy for PD is the use of direct striatal DA-ergic receptor agonists. This approach offers several potential advantages over L-DOPA therapy. Most prominently these drugs do not depend on enzymatic conversion to induce an effect, and therefore do not require functional DA-ergic neurons. This characteristic may potentially make them more beneficial for treating late-stage PD. Moreover, DA receptor agonists are more selective in their action. Unlike L-DOPA that activates all DA-ergic receptor types, this class of drugs displays relative selection to receptor subtype activation. Also, their duration of action is prolonged compared to L-DOPA, and therefore offer an improved management option of dose-related fluctuations in motor state. Finally, if the free radical hypothesis of L-DOPA should prove to be correct, then direct DA receptor agonists may offer an advantage over L-DOPA by reducing endogenous DA release, as well as to lessen the need for exogenous L-DOPA (Goetz, 1990). However, all four of the currently availabe DA receptor agonsts (bromocriptine, pergolide, ropinirole, and pramipexole) may produce hallucinations or confusion (similar to L-DOPA), and may also worsen orthostatic hypotension.

The use of COMT inhibitors (since COMT and MAO are responsible for catabolising DA) is another pharmaceutical attempt for treating PD. When L-DOPA is taken orally, nearly 99% of the drug is catabolized before it reaches the brain. The drug's decarboxylation in the periphery to DA causes nausea and hypotension. The addition of carbidopa reduces the formation of DA, but increases the fraction of L-DOPA that gets methylated by COMT. The principal therapeutic action of the COMT inhibitors is therefore to block the peripheral conversion of L-DOPA to 3-*O*-methyl DOPA. This increases the plasma half-life of L-DOPA and the amount from each dose that reaches the CNS (Goetz, 1998). In double-blind trials, the COMT inhibitors, tolcapone and entocapone, reduced the clinical symptoms of "wearing-off" in patients treated with L-DOPA/carbidopa (Parkinson Study Group, 1997; Kurth *et al*., 1997). Unfortunately, the adverse effects associated with COMT inhibitors are similar to L-DOPA and carbidopa's separate use, and in addition the COMT-inhibitors may also induce hepatotoxicity. Patients receiving this drug therefore require careful monitoring.

MAO has two isoenzymes, MAO-A, and MAO-B, with MAO-B the predominant form in the striatum. Here it is responsible for the majority of DA's oxidative metabolism. Selegiline is a selective inhibitor of MAO-B that irreversibly inhibits the actions of this enzyme (Olanow, 1993). Since it is a specific inhibitor, it does not inhibit the peripheral catabolism of catecholamines, and therefore can be taken safely with L-DOPA. Only a moderate dose of selegiline should be taken, since doses higher than 10 mg daily may inhibit MAO-A.

Before the discovery of L-DOPA, muscarinic acetylcholine receptor agonists were widely used for treating PD. Although the biological basis for the actions induced by these agents are not completely understood, the most likely scenario is that they act in the neostriatum where they exert their actions on cholinergic striatal interneurons. Five subtypes of acetylcholinergic muscarinic (M1-M5) receptors have been identified thus far, with at least four being present in the striatum (Hersch *et al*., 1994). All of the currently-used muscarinic agonists show a modest anti-parkinsonian effect. This is useful for treating early PD or as an adjunct to dopamimetic therapy. All the side-effects, such as sedation and mental confusion that stem from use of these drugs, are attributed to their anticholinergic action.

Interestingly, it has been shown that amantadine, an anti-viral agent for the prophylactic treatment of influenza, has shown anti-parkinsonian effects, although the mechanism by which it achieves this effect remains unclear. It may do so by altering DA's release or reuptake, while anticholinergic mechanisms may also be involved. Moreover, the drug's effect at the level of NMDA glutamate receptors, may contribute to its anti-parkinsonian actions (Stoof *et al*., 1992). The anti-parkinsonian effects are often described as modest only, and hence the drug is often used as an initial therapy of mild PD only. Although well tolerated, its use has been associated with dizziness, lethargy, and sleep disturbances. Fortunately these side-effects are mild and usually reversible (Hardman & Limbird, 2001).

However, to date no drug or surgery-based treatment has succeeded in completely slowing down the rate of neural loss following the onset of PD. In addition, in cases where the disease has been marginally slowed-down, these have not been without their own clinical reactions, that include side-effects due to overmedication, on/off effects as well as idiosyncratic reactions. Moreover, it is important to recognise that although laboratory experiments indicate that the use of DA direct receptor agonists may offer advantages over that of L-DOPA, clinical studies have only been able to provide limited direct evidence for this. Thus it is important to experiment and identify new neurobiological targets for preventing and/or treating the disease.

6.2. Neurosurgical intervention therapy for Parkinson's disease

The neurosurgical treatment of PD has undergone a dramatic shift in focus over the past few years. Between 1950 and 1968, thalamotomy and other neuro-ablative procedures played a critical role in the treatment of PD (Jankovic, 2006). During this period, patients who suffered from severe forms of PD were left with very little therapeutic hope for improving, often dying from poor nutrition, pneumonia and aspiration, and therefore, surgical intervention was regarded as a desperate last option. In recent years ablative stereotaxic surgery, particularly techniques that target the pallidus, have received rejuvenated status as therapeutic strategies, by allowing for improved activities relating to daily living and functional independence (Lozano

et al., 1998). Another method makes use of bilateral deep-brain stimulation employing high frequency, to reduce the hyperactivity, an essential feature of the parkinsonian state, of the STN. This increases excitatory drive in the GPi and SNr, which, in turn, overinhibits the motor fibres projecting to the thalamus and brainstem. The GABAergic internal segment of the GPi of PD patients are chronically overactive in the PD brain, and therefore the technique aims to slow down both the motor thalamus and the cortical motor system to produce the slowness, rigidity and poverty of movement that characterizes the parkinsonian state (Lozano & Lang, 1998).

However, despite the optimism that surgical therapies evoke for treating PD's motor-symptoms, certain adverse effects are associated, including potentially life-threatening side-effects, such as intracranial haemorrhage and infections. In addition, they are expensive and not universally available, and they do not seem to provide superior anti-parkinsonian benefits to what can be achieved with L-DOPA (Walter & Vitek, 2004; Olanow *et al*., 2006). Considering the above, surgical treatment is regarded as the final therapeutic option after the disease has left the patient completely incapacitated and beyond the help of any other forms of medication or therapy (Lozano & Lang, 1998; Jankovic, 2006). In addition, surgical intervention is often a last resort, when a patient is left completely disabled due to L-DOPA-induced motor complications (Olanow *et al*., 2006).

Experimental therapeutics of PD also includes implantation of genetically engineered cells and stem-cells (Svendsen, 2008). However, clinical trials performed on implanted DA-secreting cells taken from the patient's own adrenal gland have yielded little long-lasting beneficial impact. Instead, patients that had received this form of surgery developed pneumonia and bladder infections related to the adrenal gland's removal (Porena *et al*., 1996).

7. Themes and objectives of the thesis

The overall objective of the current study was threefold:

i) Firstly, to investigate for possible mechanisms that might explain why PD occurs more commonly in males than in females. To address this question, a thorough search of the literature was conducted in conjunction with a mining-search of the World Health Organization's (WHO) database on adverse drug reactions in order to determine a possible relationship between estrogen and progesterone, as the most obvious underlying mechanism and symptoms responsible for the PD syndrome (Chapter 2). The search highlighted the lack of consensus with regards to the role of gonadal hormones to offer protection in the onset and development of PD (Chapter 3). Subsequent to this, experiments were performed on young rats, to compare the effects of a toxic 6-OHDA lesion on the brains and motor-behavior of these animals. The exceptional young age of the animals served to limit the extent to which gonadal

hormones can affect the final outcome. The difference in neurotrophin levels were determined between the genders, in particular nerve growth factor as a possible measure of protection against the toxic insult and correlated this with the lesion size in the striatum.

ii) Secondly, whether early life stress could predispose DA-ergic neurons to subsequent insults was investigated. For this study the conditions injecting 6-OHDA needed optimisation, with particular emphasis placed on the delivery of the insult as well as on the development of motor-function tests, sensitive to the degree of DA loss. The lesion methodology was therefore established (chapter 4 by characterising the effects of different modes of toxin-delivery (medial forebrain bundle (MFB) vs. striatum) on motor-behavior. Once the experimental conditions, including ascertaining a set of co-ordinates were established, the question relating to the impact of early life stress on neuron vulnerability could be pursued. In doing so rat pups were subjected to maternal separation between postnatal days 2-14, prior to receiving a 6-OHDA injection into the MFB on postnatal day 35. Subsequently the effects of early-life adverse events on 6-OHDA-induced abnormalities were determined (chapter 5).

iii) Statins are used in patients suffering from hyperlipidemic conditions. However, recently it has been postulated that statins may also have neuroprotective effects. In view of this promising observation, experiments were performed to confirm this neuroprotective effect in a rotenone rat model of PD. The focus of the study was on the effects it produces on mitochondrial proteins using sensitive proteomic techniques, and to relate the biochemical results to the rats' performance in sensitive motor-function tests. This was done in order to generate a better understanding of its possible mechanism of action (chapter 7). However, the study is preceded by a thorough literature review on current proteomic techniques and its applicability to PD (chapter 6).

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CHAPTER 2

ESTROGEN THERAPY AND SYMPTOMS OF PARKINSONISM: A REVIEW OF THE LITERATURE AND AN EXAMINATION OF THE WHOS' ADVERSE DRUG REACTION (ADR) DATABASE

1. INTRODUCTION

Several epidemiological studies conducted in the US and Europe report a 1.5 to 2 times higher incidence and prevalence of PD in men compared to women (Van den Eeden *et al*., 2003; De Lau *et al*., 2004; Wooten *et al*., 2004; Claveria *et al*., 2002; Benito-Leon *et al*., 2003; Tanner & Ashton, 2000; Baldereschi *et al*., 2000; Mayeux *et al*., 1995). However, in contrast, a female preponderance for PD has been reported in Japan (Kimura *et al*., 2002). Reports of a reduced anti-parkinsonian threshold dose for Levodopa in women and additional gender differences in terms of symptoms and response to Levodopa treatment have also been reported (Blanchet *et al*., 1999). However, there are numerous discrepancies in the literature regarding gender differences reported in terms of incidence, symptoms, medication effects and response to treatment (Shulman & Bhat, 2006). These include reports by some European-based epidemiological studies of a higher prevalence of very late-onset PD found in females (Bower *et al*., 1999) and lifetime fluctuations in the progressive course of PD in female patients (Sato *et al*., 2006).

The marked gender difference in the rate of developing the disease, suggest that the brain's hormonal environment achieved during adulthood may be an important point of departure in the search for explaining the phenomenon. Realization as to the important beneficial effects that sex hormones, particularly estrogen, may play in the development and maintenance of not only the female reproductive system, but also its recognized neuroprotective effects, has resulted in the pharmaceutical development of a vast variety of steroidal and non-steroidal compounds that interact with estrogen receptors. An example of this includes the contraceptive pill, which has caused a revolution in medical practice over the past 40 years (Cianci & De Leo, 2007). Globally, more than 60 million women currently receive oral contraceptive (OC) therapy (Tierney & Luine, 1997).

The many health benefits associated with the influence of exogenously applied sex hormones on endogenous sex hormone levels have led to the development of therapeutic strategies not principally aimed at regulating the menstrual cycle, as is the case with OCs, but rather to reduce the harmful effects that may follow the cessation of menstruation, that marks the onset of menopause.

Due to improvements in medical care over the past few decades, average life expectancy has increased, accounting for the population of the Western world aging at a progressive rate (Ernst & Hay, 1994). However, the average age at which menopause commences and terminates remains constant, so that women in countries that provide a high standard of health care can currently expect to live up to one half of their adult lives following the menopausal transition phase (Vliet & Davis, 1991). Menopause is a normal milestone experienced annually by at least 2 million women in the U.S. alone (Henderson, 2005). Many women are concerned about the relationship between menopause and their health due to reports that post-menopausal hormone deficiency may leave women more exposed to neurotoxic insults. It has been suggested that the reproductive female hormones estrogen, progesterone and luteinizing hormone, which rapidly decrease post-menopause, may exert protective effects within the neuronal environment, specifically against neuronal degeneration and susceptibility to environmental toxins.

In its traditional sense, HRT entails either a combination product containing both estrogen and progestin, or single entity products that contains progestin only. Estrogen was traditionally used on its own for providing HRT and not in combination with any other steroid hormone, such as progestin, which is a synthetic variety of progesterone. The progestin compound was later added to the standard hormone replacement regimen, to diminish the risk for developing endometrial and breast cancer, that seems to be associated with using estrogen on its own (Sismondi *et al*., 1999). Non-traditional HRT emphasizes the benefit of combining a variety of hormones, for orchestrating their overall effects on energy levels and general well-being. These therapies comprise of metabolic precursors of DHEA (dehydroepiandrosterone) and pregnenolone, as well as various combinations of growth hormone, melatonin, and the thyroid hormones. The addition of an androgen to the therapeutic regime (usually testosterone), has proved useful for restoring lowered energy levels, libido and to prevent osteoporosis during post-menopause (de Paula *et al*., 2007; Taelman *et al*., 1989).

Various gynecological benefits are associated with HRT. This includes the ability to reduce the incidence of secondary amenorrhea (Romer *et al*., 2000), prevent benign breast neoplasms (Kramer *et al*., 2006) and anaemia due to an iron-deficiency (Grimes, 1992), reduce the risk of endometrial cancer (Chubak *et al*., 2007; Kelsey *et al*., 1982; Schlesselman, 1997; Weiderpass *et al*., 1999), prevent ectopic pregnancies (Sivin, 1988; Sivin, 1991; Franks *et al*., 1990), diminish the risk for developing functional ovarian cysts (Peterson *et al*., 1997), and reduce the incidence of pelvic inflammatory disease (Lanes *et al*., 1992).

However, valid concerns have been raised that the substantial hormonal changes associated with natural or surgically induced menopause may have adverse reactions and contribute towards the initiation and/or development of various diseases. For example, several

studies have indicated a significantly higher incidence of strokes, due to thrombo-embolism, subarachnoid hemorrhage and cerebral venous thrombosis in users of oral contraceptives, particularly in those receiving high estrogen formulations (Vickers *et al*., 2007).

Of the list of active chemical components contained in the most commonly prescribed HRTs, estrogen has received the largest blame for adverse effects on systems and organs not directly associated with reproduction. At the same time, it has also received the greatest amount of praise for its beneficial influence, most notably that of protecting against disease-processes, such as against cardiovascular and various neurological disorders, including PD (Gaspard *et al*., 2002).

In animal models of PD, an apparent neuroprotective effect was exerted by estrogen when it was administered prior to or coinciding with the toxic insult that simulates symptoms of PD. This was successfully demonstrated using methamphetamine (Gao & Dluzen, 2001; Gajjar *et al*., 2003), while estrogen's neuroprotective benefit was shown with the use of the neurotoxin MPTP in primates (Dluzen *et al*., 1996; Miller *et al*., 1998). Beneficial effects were also reported for the 6-OHDA rat model (Dluzen, 1997). Furthermore, a loss of dopaminergic neurons in the SN of non-human primates exceeding 30% due to estrogen deprivation has been reported (Leranth *et al*., 2000), while chronic estrogen replacement restored dopaminergic function in ovariectomised rats (Ohtani *et al*., 2001).

Estrogen exerts its effects on the CNS via both genomic mechanisms, modulating the synthesis, release and metabolism of neurotransmitters, neuropeptides and neurosteroids, as well as through non-genomic mechanisms, by influencing electrical excitability, synaptic function and morphological features. These influences may not only exert an influence on the male preponderance observed in a disease associated with the nigrostriatal dopaminergic system, such as PD (Morale *et al*., 2006), but may also be pivotal in the pathogenesis of other age-related neurodegenerative diseases, such as AD (Benedetti *et al*., 2001). However, the evidence lacks consistency: HRT was found to protect against developing AD when administered post-menopause (Morinaga *et al*., 2007; Hogervorst *et al*., 2000), while a large, randomized, double-blind, placebo-controlled clinical trial of postmenopausal HRT failed to replicate these findings (Mulnard *et al*., 2000).

It remains uncertain what estrogen's mechanism of effect on the CNS involves (Van Hartesveldt & Joyce, 1986; Kompoliti, 1999). As far as PD is concerned, studies done using animal models of the disease indicate that estrogen affects the synthesis, release and metabolism of DA via its effects on DA uptake sites (Becker, 1990). It further showed that estrogen may also modulate DA receptor expression and function (Di Paolo *et al*., 1988), thereby influencing the behavior normally regulated by the basal ganglia (Roy *et al*., 1990). Whereas clinical studies have also reported beneficial effects of estrogen in providing

symptomatic relief to PD patients (see Schipper, 1988, for a review), some investigators report only a slight anti-parkinsonian benefit in response to 17ß-estradiol therapy (Tsang *et al*., 2000).

Lyons *et al.* (1998) made use of a 'neuroendocrine challenge', which is a technique used for exploring neurotransmitter tone in humans, providing an indirect means of detecting the effects of estrogen on the dopaminergic response. The investigators used an apomorphine-induced growth hormone (GH) secretion delivered *via* post-synaptic D_2 receptors, located in the median eminence of the hypothalamus (Meltzer, 1979), to investigate central dopaminergic responsivity in female patients. The results from this study indicate that post-menopausal women, who undergo long-term estrogen therapy, have enhanced GH responses, compared to women who are naïve to estrogen therapy, thereby suggesting increased dopaminergic tone, and less likelihood for developing PD. Further support for estrogen's influence on brain function derives from experiments showing that ovarian steroids may regulate the expression of the dopamine-degrading enzyme catechol-*o*-methyltransferase (COMT) (Di Paolo, 1994; Becker, 1999; Xie *et al*., 1999), while it was also shown that estrogen could also act directly on the striatum (Van Hartesveldt & Joyce, 1986).

Clinical studies performed on PD patients have also illustrated that estrogen may have behavior-mediating effects on DA, due to the influences it exerts on the basal ganglia (Hruska & Silbergeld, 1980), with biochemical evidence to suggest that estrogen regulates dopaminergic neurotransmission in the basal ganglia (Di Paolo *et al*., 1988; Becker, 1990). However, inconsistent reports are the norm rather than the exception. Studies to investigate whether estrogens stimulate or inhibit the human dopaminergic system include reports that the use of estrogen on its own (in the absence of progesterone) may increase the risk of developing PD among women who have undergone a hysterectomy (Marder *et al*., 1998), observing a greater decline in the D_2 dopamine receptor concentration in women (Wong *et al.*, 1988). In addition, the complete absence of modifying effects (Currie *et al*., 2004; Strijks *et al*., 1999) has also been observed.

These inconsistencies are difficult to explain, especially in light of evidence suggesting that neither dopamine-sensitive cells located in the originating mesencephalic region, nor those in the basal ganglia show the ability to concentrate estrogen (Van Hartesveldt & Joyce, 1986). These findings add to the confusion and provide inadequate foundations for practice guidelines. The uncertainty as to whether PD is provoked or suppressed when the underlying pathology comes in contact with the active components of HRT is worrisome, especially since, due to an aging population, an increasing number of people can expect to experience age-related disorders associated with abnormalities of the dopaminergic system, that includes PD (Tanner & Ashton, 2000). In the absence of investigations to establish our insight into the effects that estrogen exerts on the dopaminergic system, especially in terms of long-term therapeutic

influences, the general regard is that the beneficial results gained from HRT significantly outweigh the risks involved, and HRT continues to gain widespread prescriptive support. The current article evaluates the apparent paradox of the effects of estrogens on the CNS with particular reference to whether the provision of HRT will worsen or reduce the chance of developing PD or PD-like symptoms, or alter specific attributes of the PD syndrome.

2. METHODS

The present chapter reviews currently available data to assess the relative safety supplementary hormone therapy offers to women. The rationale for this is that the number of women reaching their midlife years and beyond who seek professional advice regarding HRT is likely to double. Yet the studies that have investigated the relative safety of estrogen treatment in the context of developing PD-related symptoms are limited. At best, and as pointed out, the studies are inconclusive with findings to suggest both an associated risk (Morale *et al*., 2006; Currie *et al*., 2004), as well as no risk associated for developing the disease (Gaspard *et al*., 2002). In view of this contradiction, I revisited existing published data, in conjunction with the WHO Programme for International Drug Monitoring, based in Uppsala, Sweden, to examine the increased risk that HRT may specifically hold for developing PD-related symptoms. The programme's database contains approximately 3.7 million case reports of suspected adverse drug reactions that have been filed as reports since 1968. Eighty-one countries in total currently participate in the scheme, with these spontaneous reports providing a first-line surveillance method for the detection of new adverse drug reactions (ADRs). The database is extremely useful because of the sizable population surveyed and the prompt reporting of unexpected adverse reactions (Olsson, 1998).

A data-mining search (Smith *et al*., 1996) was performed to search for drug-ADR combinations occurring more frequently than expected. A positive drug-reaction association implies a significant difference from the entire global report (Bate *et al.*, 1998). The search was done using the anatomical therapeutic chemical (ATC) grouping to cover all estrogen-containing compounds. Reports with drugs belonging to any of the following ATC groups were included in the search: Hormonal contraceptives for systemic use/progestogens and estrogens; estrogens/natural and semisynthetic estrogens; synthetic estrogen; estrogen combined with other drugs; androgens and estrogens; androgen alone; progestogen and estrogen in combination; and progestogens and estrogens in combination. The following WHO-preferred terms were used during the search for therapy-associated movement disorders: Bradykinesia, Dyskinesia, Hypokinesia, Choreoathetosis, Hypertonia, Tremor, Extrapyramidal disorder and Parkinsonism aggravated. Three subterms were included under the preferred term

'extrapyramidal disorder', namely 'Parkinson's syndrome', 'Parkinsonism' and 'Parkinsonian gait'.

3. RESULTS AND DISCUSSION

Very few reports of PD per sè were revealed during the search, to suggest that PD is either an unrecognised adverse effect of estrogen-containing compounds or that only a weak relationship exists between the two. However, it is acknowledged that under-reporting and failure to recognise adverse reactions with a long latency are two weaknesses inherent to this particular reporting system. A disproportionately high amount of chorea-cases was reported, compared to all other drugs in the database, though this does not reach significance at the 95% confidence level. Differential diagnoses of chorea, in addition to Sydenham's chorea, include Wilson's disease; encephalitis; Huntington's chorea; drug intoxication; benign familial chorea; pregnancy; systemic lupus erythematosus; Henoch-Schonlein purpura; polycythemia vera; hypocalcemia; hyperthyroidism; carbon monoxide poisoning; cerebral infarction and intracranial tumor (Dove, 1980). It involves rhythmic motor oscillations characterized by brief, irregular muscle contractions that are not repetitive or rhythmic in nature, but rather appear to continuously flow from one muscle to the next, often co-presenting with athetosis, that adds twisting and writhing movements by the hands, feet, trunk, neck and face, to the already existing disorder. Drug-induced chorea is the most commonly occurring type found in clinical practice (Cardoso *et al*., 2006; Wenning *et al*., 2005). More than 40% of all PD patients, depending on age and the dosage used (Fahn, 2000; Schrag & Quinn, 2000), develop chorea after 5-10 years of continued Levodopa use, with increasing numbers of patients affected as time progresses (Obeso *et al*., 2000). It is thought that chorea is a side-effect to the drug's ability to stimulate postsynaptic DA receptors in a pulsatile fashion, evoking changed responses amongst the neuronal networks that interconnect the basal ganglia with the frontal cortical motor regions, with such excessive thalamocortical facilitation overactivating the release of DA (Fahn, 2000). However, inconsistencies between the proposed model and clinical evidence have been observed, including the absence of increased excitatory thalamocortical drive following pallidotomy, which should worsen the chorea according to most hypotheses explaining the origin of the phenomenon (Liu *et al*., 2006). In light of this, current opinion on what constitutes hyperkinetic movement disorders, such as chorea, maintain that more complex alterations in the temporal and spatial firing pattern of the globus pallidus might be responsible for this clinical phenomenon (Liu *et al*., 2006; Obeso *et al*., 2002; Moro *et al*., 2004). For instance, it has been postulated that chorea may be due to estrogen's direct action on DA, or, alternatively, may result from its competitive binding to COMT (Schipper, 1988).

Following the first report of an association between chorea and OCs by Fernando in 1966, various additional case-reports and epidemiology-based studies have revealed chorea to be a rare complication of estrogen-containing oral contraceptive-therapy, such as the use of topical vaginal cream containing conjugated estrogen (Caviness & Muenter, 1991). One large-scale study reported that 12% of all patients that participated in the study, developed chorea soon after starting estrogen-containing oral contraceptive treatment, with 6% developing chorea gravidarum and 2% developing the disorder shortly following delivery of a baby (Cervera *et al.*, 1997). In contrast to other drugs, that may also lead to the development of chorea as a side-effect to their use, OCs require the presence of an already existing basal ganglia dysfunction in order to induce its adverse effects (Miranda *et al*., 2004). Such adverse drug reactions are only expected to occur as recurring choreic episodes, such as in Sydenham's chorea, chorea due to systemic lupus erythematosis, chorea gravidarum (Karageyim *et al*., 2002) or when Levodopa-induced (Fahn, 2000). The time between initiating therapy and the appearance of choreiform movements vary between 6 days to 9 months, with 3 months generally regarded as the mean norm (Dove, 1980). This supports previous reports that female PD patients present more often with tremor than males (Haaxma *et al*., 2006), with tremor regarded as a mild symptom of striatal degeneration.

4. CONCLUDING REMARKS

There are several ways in which human studies could be performed with different comparisons made between genders, age groups, with either the use of HRT or not, as well as by carefully differentiating the components of PD. Recent reports of a correlation between estrogen-based therapy and chorea were found to lack in recent literature, and large studies on PD with sufficient power to examine the differential effects of estrogen on the PD syndrome have not been done. However, the current review highlights that there is indeed cause for concern in this regard, because of the conflicting results presented, with a definite need for further investigation in order to extrapolate for the relationship between female gonadal hormones and the nigrostriatal dopaminergic system. The absence of evidence for any signal on the adverse effects of estrogen containing compounds on PD in the WHO database could be interpreted as a failure to recognize a signal for a causative effect or worsened PD, even though the database revealed chorea as a side effect to estrogen-based therapy. However, the result does not conflict with the possibility that estrogens may protect from all or some aspects of the PD syndrome. Intensified research efforts are called for before agreement can be reached with regards to the locus, direction, or the mechanism of OC's and HRT's action on basal ganglia function.

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CHAPTER 3

EARLY PUBERTAL FEMALE RATS ARE MORE RESISTANT THAN MALES TO 6-HYDROXYDOPAMINE NEUROTOXICITY AND BEHAVIOURAL DEFICITS: A POSSIBLE ROLE FOR TROPHIC FACTORS

1. INTRODUCTION

Gender-related differences may play a role in the patho-etiology of PD, since such differences have been detected in terms of the prevalence, symptomatology, prognosis, and age of onset of the disease. Although the precise biological mechanisms remain to be determined, findings from several epidemiological studies indicate that, in general, men are more frequently affected and display a younger age of PD onset than women (Baldereschi *et al*., 2000; Bower *et al*., 1999; Kuopio *et al*., 1999; Mayeux *et al*., 1995; Milanov *et al*., 2001; Schrag *et al*., 2000). Reports from several basic studies provide further support for this phenomenon, and suggest that biochemical and functional gender differences inherent to the DA pathway may underlie the male prominence seen in PD (Becker, 1990; Becker, 1999; Castner *et al*., 1993; Murray *et al*., 2003; Tamas *et al*., 2005). A neuroprotective effect of the gonodal steroid hormone estrogen has been demonstrated in animal models of PD that involves the administration of DA neurotoxins (Miller *et al*., 1998; Sa & Madeira, 2005). A number of studies support the concept that estrogen may play a role in the synthesis (Pasquali *et al*., 1995), metabolism (Xie *et al*., 1999) and transport of DA (Disshon *et al*., 1998), suggesting that an intimate interaction exists between estrogen and the DA-system. Moreover, the estrogen receptor, ER*β*, localizes to mitochondria in a variety of cell types, including neurons, cardiomyocytes and a murine hippocampal cell line (Yang *et al*., 2004). Mitochondria generate most of the cell's ATP by means of oxidative phosphorylation, in addition to endogenous oxygen radicals, as its toxic by-product (Brookes *et al*., 2002), and fulfill a central role in regulating apoptosis, calcium homeostasis and a cytoplasmic redox state (Chakraborti *et al*., 1999; Hengartner, 2000). Although the molecular mechanisms that lead to neuronal cell death in PD remain elusive, studies done on post-mortem human brain, genetic studies of familial PD, and toxin-based animal models of PD, all suggest that a common pathway involving mitochondrial dysfunction may be involved in dopaminergic neuron degeneration (Liang *et al*., 2006; Ramsey *et al*., 2007; Song *et al*., 2004; Yang *et al*., 2006).

Several studies suggest that estrogen has either direct or indirect effects on mitochondrial function (Mattson & Magnus, 2006; Nilsen & Brinton, 2003; Olanow *et al*., 2003; Wang *et al*.,

2001), indicating that mitochondrial ER*β* has a mediating role on the effects estrogen has on mitochondrial functions. This may have implications concerning DA neurotoxins that target mitochondria, as well as for gender-related effects on the manifestation of PD. The male gonadal steroid hormone testosterone apparently provides no protection for DA neurons against neurotoxic agents (Dluzen *et al*.,1996; Ekue *et al*., 2002; Gao & Dluzen, 2001), while certain reports even suggest that testosterone may enhance neurodegeneration (Dluzen & McDermott, 2002; Dluzen & Horstink, 2003; Gillies *et al*., 2004; Horstink *et al*., 2003). The search for novel forms of therapy against PD has recently undergone a remarkable shift from developing drugs that merely relieve the intensity of PD symptoms experienced, to a new generation of remedies that can potentially protect DA neurons as well as to modify the course of the disease. In addition to the possible neuroprotective effect of estrogen, other neuroprotective agents have displayed gender-specific effects also. This includes pituitary adenylate cyclase activating polypeptide (PACAP) (Reglodi *et al*., 2006), which was found to have no effect on DA cell survival in intact females, while effectively increasing DA cell survival and decreasing behavioural deficits in ovariectomized females.

Neurotrophic factors, and their respective receptors, are key regulators during development and maintain a homeostatic state within the CNS environment. Neurotrophins, a neurotrophic subclass, has been earmarked for potential therapeutic applications in neurodegenerative diseases on the basis of results obtained from *in vitro* experiments and in animal models. Recent studies have demonstrated the presence of increased trophic factor levels in the striatum following a nigrostriatal lesion (Ling *et al*., 2000). Although experimental focus has rather been on the differential expression of other factors belonging to this trophic class of agents, Nerve Growth Factor (NGF), although having received less attention than its trophic counterparts, including Glial Derived Neurotrophic Factor (GDNF), Brain Derived Neurotrophic Factor (BDNF) and Neurotrophic-3 (NT-3), is a factor highly regarded for its antioxidant (Castner *et al*., 1993), anti-apoptotic (Shimoke & Chiba, 2001) and neurorescuing properties. As a universally applicable molecule, it controls path finding, spatial patterning, proliferation, as well as to provide protection for the cells of the CNS (Stromberg *et al*., 1990). These properties may be especially relevant to PD, since it has been shown that NGF concentrations are decreased in PD patients and in experimental parkinsonian rats (Lorigados *et al*., 2002). This is particularly the case in systemic administration of rotenone, an agricultural pesticide used for its capacity to selectively degenerate nigral DA neurons and mimic PD-like symptoms in rats (Betarbet *et al*., 2000). NGF was shown to significantly reduce rotenone's toxic effects on neurons in midbrain neuronal cultures, by staining more positively for tyrosine hydroxylase (TH) (Jiang *et al*., 2006), the rate-limiting enzyme involved in catecholamine biosynthesis (Glinka *et al*., 1997; Kobayashi & Nagatsu, 2005). One of the most commonly used experimental models of PD involves the central administration of the

neurotoxin 6-OHDA, or 2, 4, 5-trihydroxyphenylethylamine, a structural analogue of both DA and noradrenaline (Senoh *et al*., 1959). It acts by means of a complex mechanism of toxicity, which involves a variety of biochemical features, including alkylation, rapid auto-oxidation and the impairment of mitochondrial energy production (Brookes *et al*., 2002; Glinka *et al*., 1997). It is generally used to experimentally induce a relatively selective lesion of neurons in the NSDA pathway (Schwarting & Huston, 1996), similar to the damage seen during post-mortem investigations of the brains of PD patients. 6-OHDA has been found present in the basal ganglia in humans, and urine samples indicate that its concentration increases in response to levodopa therapy (Andrew *et al*., 1993). A useful feature of 6-OHDA is its capacity to create asymmetrical DA neurotransmission between the lesioned and the intact hemisphere in animals receiving unilateral drug administration. This leads to marked impairment of normal motor function in the limbs opposite to the lesion side, resulting in the preferential use of the limbs ispsilateral to the lesioned hemisphere. Interestingly, the exogenous application of NGF has been shown to increase the degree of antioxidant enzymes, heme oxygenase-1 and prevent apoptosis by possibly triggering an anti-apoptotic gene-signaling mechanism following exposure to 6-OHDA (Salinas *et al*., 2003).

In the current study, we made use of gonad-intact animals, since limited studies have made direct male-female comparisons (Miller *et al*., 1998; Yu & Liao, 2000; Zhang *et al*., 1998). We specifically opted to work with adolescent rats, since the synthesis-rates of striatal D_1 and D_2 DA receptors reach their apices at this stage with gender differences diverging thereafter (Andersen, 2002) and since it has been shown that estrogen is only present in the brain after the second month of life (Knoll *et al*., 2000). Emerging evidence also indicates that key gender differences in gene expression and biochemistry in SN TH positive neurons do not depend on gonadal sex steroids (Dewing *et al*., 2003; Dewing *et al*., 2006). Gender differences were found in behavioral impairment tests, and these correlated with differences in the degree of TH-staining and NGF expression following 6-OHDA administration.

2. METHODS AND MATERIALS

2.1. Animals

Sprague-Dawley rats were used throughout the study. Twelve animals were used per group (dosage and gender). The animals were kept in clear 40 × 25 × 20 cm Plexiglas cages, in an AAALAC accredited facility, in rooms with temperature 22 ± 1◦C and a 12:12 hr. light:dark cycle (lights on from 07h00 to 19h00). The rats had *ad libitum* access to pelleted rat chow and tap water. All experimental procedures followed in this study had been approved by the Committee for Experimental Animal Research of the University of Stellenbosch (Project

number: N05/04/004), and were in accordance with the National Institutes of Health Guide for care and use of laboratory animals.

2.2. Preoperative and surgical procedures for the infusion of 6-hydroxydopamine into the MFB

Initial experiments were performed to establish a set of medial forebrain bundle (MFB) coordinates since information on 6-OHDA injections into the MFB of young animals was very limited. For these lesions, Indian ink was injected into the brains of a group of animals at 35 days of age, in order to ascertain the optimal location of the tip of the injection cannula (30G needle). A combination of ketamine hydrochloride (Anaket-V, Bayer Healthcare, South Africa) and medetomidine hydrochloride (Domitor, Pfizer, South Africa) (0.1 ml/100g, i.p.) was used to anaesthetise the animals. It is acknowledged that ketamine hydrochloride may possibly provide a degree of neuroprotection to the animals during surgery. However, most experimental studies have failed to show effective long-term protection, despite impressive short-term protection offered (Koerner & Brambrink, 2006). Furthermore, although ketamine protected the cellular integrity in striatal slices in culture, it did not affect the neurotransmitter release of these cells (Becker, 1999) and the anaesthetic agent also failed to alter the results of neurobehavioural outcome tests performed on patients at either one or ten weeks post-intervention (Nagels *et al.*, 2004). Moreover, since all animals were treated with the same dosage regime, it can be assumed that all groups were exposed to a potentially similar degree of neuroprotection and are thus comparable from this perspective. Following the anaesthetic injection, the animals were placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). To prepare the surgical site, hair was clipped and the site disinfected with 70% Alcohol (EtOH). The skin was sterilized using an iodophor (Betadine, Purdue Pharmaceutical Products). A rubber heating pad (REX C10, RKC, 19.0 ± 99.9◦C heat generator, Electronic Services, University of Stellenbosch) maintained the rats' body heat during surgery. Following the Indian ink injection, the rat was decapitated and the brain placed in paraformaldehyde for 48 hrs. The tissue was cut with a cryostat at a thickness of 30 μm. The sections were mounted onto clear glass slides and examined under a Nikon TE2000-E microscope (using a phase 4 resolution) to determine the location of the lesion, while referring to a stereotaxic atlas of the rat brain (Paxinos & Watson, 1986). The stereotaxic coordinates for the MFB injection established were: 2.5 mm posterior to Bregma, 1.8 mm lateral to the midline and 7.8mm below the dura. These coordinates were used throughout the study for 6-OHDA and sham lesioned rats. For a photographic image of the site of needle placement, see Fig. 1. Prior to surgery 35 day-old experimental animals, weighing between 110–142g were randomly assigned to either one of the dosage-groups or the control-group. The mean

weights of the male rats were comparable to those of the female rats at this young age. In accordance with Roberts *et al*. (1975), rats were injected with 25 mg/kg desipramine (i.p.) thirty min before and immediately after the operation to limit concurrent damage to noradrenergic pathways by 6-OHDA infusion. A free-base weight of 6-OHDA (Sigma, St. Louis, MO) of 1 µg for the low dose and a higher dose of 15 µg was dissolved in 4 µl 0.1% ascorbate in 0.9% sterile saline and unilaterally (right MFB) injected. The solution was infused over a period of 5 min using a 341-A model syringe pump (Sage Instruments, USA). Following the infusion, the needle was left in place for an additional 5 min, before slowly retracting it from the brain. The burr-hole drilled through the skull was filled with sterilized oxidized cellulose (Ethicon, UK), while the incision was sutured and again coated with iodophor. To recover, the rats were placed in individual cages under lamps to provide adequate heating. The condition of the sutures, the rats' food and water intake, and their behaviour were monitored daily to ensure full recovery.

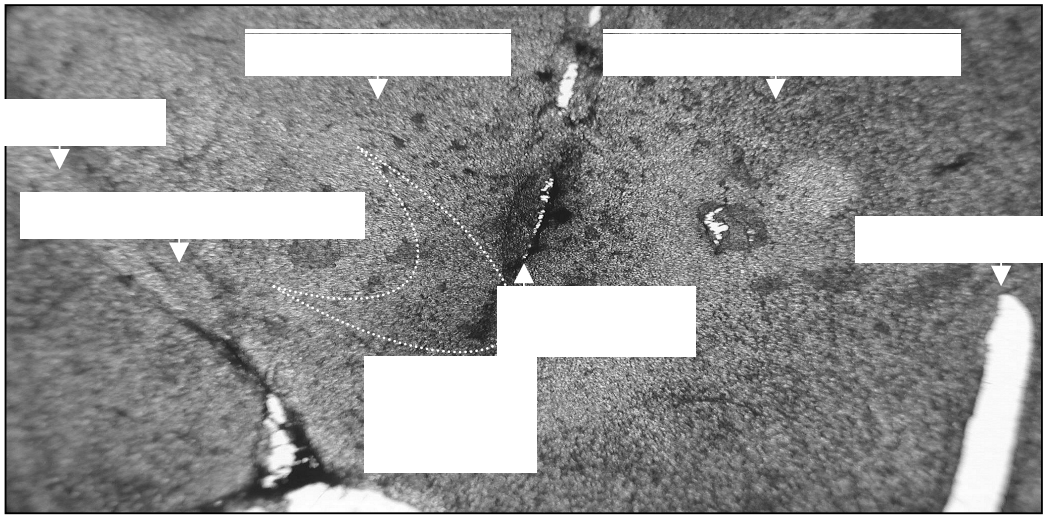

Fig. 1. An image showing the placement of the needle into the MFB, for the injection with 6-OHDA. The image was obtained with a Nikon TE2000-E microscope, the objective lens being × 4 with a Brightfield application attached, while the image was captured with a Sony 3CCD camera that was mounted upon the microscope.

2.3. General behavioral testing procedures

Animals were tested for behavioral changes at 14 days post MFB-injection. Testing consisted of (1) the adjusting-steps test (Olsson *et al*., 1995; Schallert *et al*., 1992), and (2) the

vibrissae-evoked forelimb placing test (Anstrom *et al*., 2007; Woodlee *et al*., 2005). All testing took place between 10h00 and 12h00.

2.3.1. Adjusting-steps test

The rats were monitored for their ability to make postural adjustments in response to an imposed weight-shift by using an isolated forelimb test (initially described by Schallert *et al*. (1979; 1992). The test is useful for monitoring lesion- and transplant-induced changes in forelimb motor function, which is a behavioral parameter that may be analogous to limb akinesia and gait problems seen in patients with Parkinson's disease (Olsson *et al*., 1995). Briefly, the experimenter suspended the rat above a tabletop surface, with the hind limbs and one forelimb restrained in one hand. This allowed the rat to support most of the weight of its body with the unrestrained forelimb only. While the experimenter slowly moved the animal at a constant rate (300 cm in 30 sec), the number of adjusting steps taken by each forelimb was recorded by the experimenter, who was blind to the treatment of the rat. This procedure was performed across two conditions, namely when the animal was moved along the table-surface in the lateral forward direction (leftward movement), as well as in the lateral backward direction (rightward movement). Two trials for each animal were performed with each trial consisting of the rat being moved once in each direction. The mean score of the two trials was considered a representative result for each animal.

2.3.2. Vibrissae-evoked forelimb placing test

In this test, which requires integrated motor and sensory input, the experimenter held the rat by the torso, thereby allowing the forepaw being tested to hang freely while the untested forelimb was gently restrained. Each forelimb was tested independently by orienting one side of the animal towards a tabletop and moving the animal in a slow lateral direction toward the edge until the vibrissae of that side made contact with the table's edge (Barth *et al*., 1990; Schallert & Tillerson, 1999). In an intact animal the stimulated vibrissae will cause the rat to instinctively place both forelimbs onto the tabletop, while, with lesioned animals, placing may not occur at all (scoring '0'), indicating a failed motor response towards a spatio-sensory stimulus, or there is a delay between stimulation and placing. Ten trials of each forelimb were performed during the testing session. An experimenter, blind to the experimental condition of the animals, recorded the number of successful placing reactions by each forelimb.

2.4. Animal euthanasia and tissue collection

Following the behavioral assessment on PND 49, the rats were decapitated and the left and right striatum rapidly dissected on ice. The tissue was immediately frozen in liquid nitrogen and stored at −80◦C until NGF levels were determined at a later stage. For assessing the degree of TH-staining, a group of anaesthetised animals (*n* = 12) were transcardially perfused with 200 ml of saline followed by 200 ml of 10% paraformaldehyde. Immediately after this, the brains were removed and placed into a solution consisting of 30% sucrose dissolved in phosphate buffered saline (PBS) for 48 hrs. The selection of animals for either the NGF assay or TH-staining was performed randomly, with only the group that had been subjected to the higher dose of 6-OHDA being considered for assessing the degree of TH-staining.

2.5. Nerve Growth Factor (NGF) assay

The Emax ImmunoAssay System, purchased from Promega (Madison, USA) was used to determine striatal levels of NGF. The striata on both the left and right (lesioned) side were used to determine whether the observed behavioural changes were mirrored by changes in neurotrophin expression. Tissue was weighed and then freshly prepared buffer (20 mM Tris-HCL (pH set at 8), 137mMNaCl, 1% NP40, 10% glycerol, 10 *μ*g/ml aprotinin, 1 μg/ml leupeptin, 0.5 mM sodium vanadate (NaVO4) and 1 mM phenylmethylsulfonyl fluoride (PMSF), was added. The samples were sonicated for 20s. The lysates were centrifuged for 20 min at 9.66 × *g* at 4◦C and the supernatant removed and stored at -20*o*C overnight. The NGF assay was subsequently performed in a 96 well plate as specified by the manufacturer. Results were expressed as pg of NGF per mg wet weight (ww) of tissue.

2.6. Tyrosine hydroxylase (TH) immunohistochemistry and quantification of lesion size

Free-floating coronal sections of a thickness of 30 μm were cut with a cryostat at −20◦C for subsequent TH-staining. The sections were quenched by incubating with 0.3% hydrogen peroxide for 30 min at room temperature, in a humidifying container followed by a succession of washes (5 × 10 min) with PBS (0.15 M, pH 7.4). The sections were placed in Eppendorf containers, each containing 10 mM citrate buffer (pH 6.0), and boiled for 5 min in a pot of boiling water standing over a heat source. The sections were left to cool down to room temperature, before the wash-cycle was repeated. Sections were pre-treated with 150 μl horse blocking serum and 0.1% Triton X-100 for 30 min, before primary monoclonal anti-TH (BrdU mouse monoclonal, dilution 1:500, DiaSorin, USA) was applied. The sections were incubated overnight at 4◦C. The next day, the slices were washed in PBS (5 × 10 min), and the secondary biotinylated link antibody (dilution 1:8000) applied for 1 hour at room temperature. The wash routine was repeated, before the slices were incubated with

peroxidase labeled mouse IgG ABC reagent. Immunostaining was performed with an ABC kit, purchased from DakoCytomation (USA), with concentrated diaminobenzidine (DAB) used as the chromogen. Tissue sections were incubated at room temperature until staining developed. The sections were given a final 15 min rinse with distilled water, before dehydrating them with 96–100% alcohol (EtOH), cleared with Xylol (Merck, South Africa) and finally mounted onto clear glass slides. Sections from six animals from each gender group were analyzed. Digital photographic images of all the slices were captured under unchanging, unchanging, standardized conditions of lighting and exposure using the 20 × objective on a Zeiss microscope fitted with Brightfield optics and a Diagnostic Instruments Spot Insight QE camera. For each hemisphere, 3 independent images were taken. Sections representing the anterior (plate 14; Paxinos & Watson, 1986), middle (plate 18; Paxinos & Watson, 1986) and posterior (plate 22; Paxinos & Watson, 1986) striatum from 6 female and 6 male animals were analysed. This was done to ensure that equivalent sections were analyzed. TH-staining density of each striatal image was determined with Zeiss's Axiovision image analysis software. Each image of the striatum was outlined with a cursor and the software analysis program was used to compute the total surface area that stained positively for TH. The combined TH-immunoreactive striatum surface area of all 3 images in the 6-OHDA infused hemisphere was divided by that of the striatum in the non-infused hemisphere. The ratio between TH-immunoreactivity on the lesioned side to TH-immunoreactivity on the non-lesioned side that acted as the control condition was then expressed as a percentage. Therefore, values closer to 100 indicate that there were areas of TH-immunoreactivity on each side that were comparable while values closer to zero indicate severe loss of TH-positive terminals. The results for each animal were averaged and the result used to obtain a mean value for all 6 female and 6 male rats. The percentage loss of TH-staining in the lesioned striatum was taken to indicate the degree of neurochemical asymmetry for that animal.

2.7. Statistical analysis

The behavioral data was statistically analysed for multiple comparisons against a single control group by means of an analysis of variance (ANOVA), with the between-subject factors being dosage-group and gender. Simple comparisons between two groups, such as the data from the NGF assay and TH-staining were performed using a Student's unpaired *t*-test. *Post hoc* comparisons between groups were conducted when appropriate using a Bonferroni test. The statistical analysis of all the data was conducted using Statistica software (StatSoft,

Tulsa, OK). Differences were considered significant when $p < 0.05$. Each data point presents the mean value ± S.E.M.

3. RESULTS

3.1. Tyrosine hydroxylase (TH) immunocytochemistry

Fixated brain slices collected from animals treated with the higher dose of 6-OHDA were analysed for the area that immunohistochemically stained positively for TH. The level of TH-staining, expressed as a ratio between lesioned and non-lesioned hemispheres, was found to be significantly decreased (p <0.05, t =8.26, df = 10) in the males compared to the females (Fig. 2).

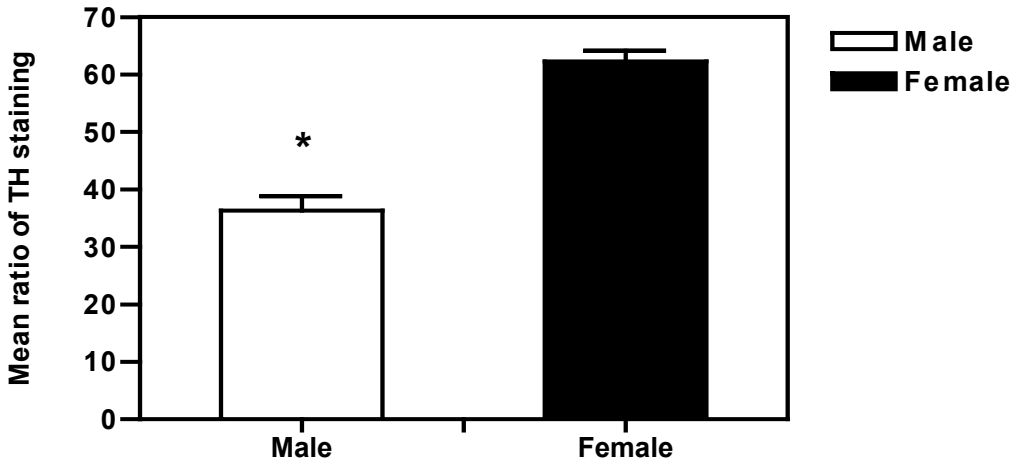

Fig. 2. Percentage TH expression in striatal sections of the 6-OHDA infused hemisphere of male and female rats. The percentage staining in the toxin-infused hemisphere of the males were significantly different from that of the females. Both gender-groups had received the higher dose of neurotoxin; *$p < 0.05$. Data represent the mean ± S.E.M. (n = 6).

3.2. Nerve Growth Factor (NGF) assay

A 2 (gender) × 3 (dose) × 2 (hemispheres) factorial design was used to analyze data obtained from the NGF assay. A significant main effect was detected for dosage (F = 18.2, df = 2, p < 0.01) as well as for gender (F = 20.54, df = 1,p < 0.01), but not for hemisphere (F = 1.92, df = 1, p = 0.17). Post-hoc comparison detected a significant interaction between dosage and gender (F = 17.4, df = 2, p < 0.01), but not between dosage and hemisphere (F = 1.3, df = 2, p = 0.29), or between gender and hemisphere (F = 3.13, df = 1, p = 0.08). No significant overall effect was found between dosage, gender and hemisphere (F = 0.96, df = 2, p = 0.39). *Post hoc* analysis revealed significantly higher levels of NGF (p < 0.01) in the female rats treated with the higher dose of 6-OHDA compared to all other groups (Fig. 3).

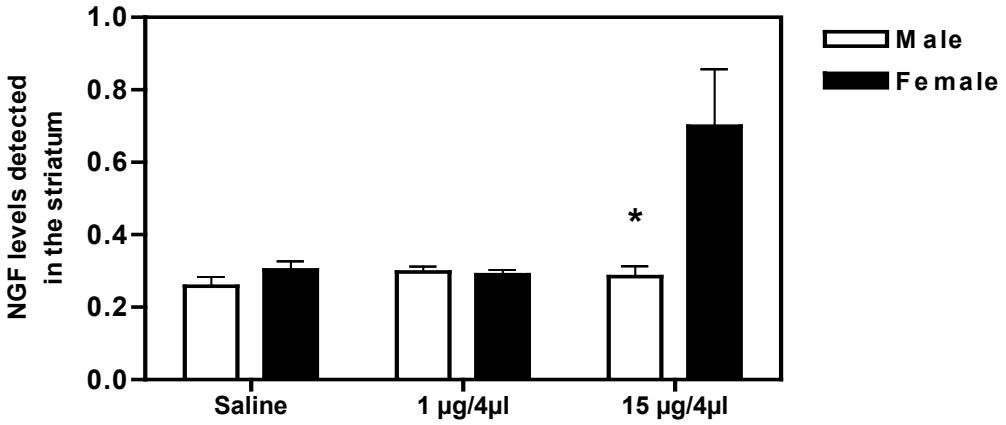

Fig. 3. A graph showing the average amounts of NGF found in the striatum tissue on the hemispheric side that had been injected with 6-OHDA. The graph shows the results of the rats subjected to two doses of 6-OHDA in addition to the group injected with the saline vehicle only. The * symbol indicates a significant ($p < 0.05$) difference from the male rats.

3.3. Behavioral tests

3.3.1. Adjusting-steps test

At 14 days post-lesion, male rats were significantly more impaired than females in their capacity to make adjusting steps in response to experimenter-imposed lateral shifts of weight following the higher, but not the low 6-OHDA dose (Fig. 2). Data were analysed using a cross-classification ANOVA. This included the between-factors: gender (2 levels: male and female), dosage-treatment (3 levels: a saline-control group, 1 µg/4 µl and 15 µg/4 µl of 6-OHDA), direction of movement (2 levels: forward and backward), and the side that was allowed to make contact with the table-surface during the trial (2 levels: left and right forelimb). No significant 4-way interaction was found between the factors dosage, gender, direction and forelimb-side ($F_{(2.48)} = 1.48$, $p = 0.23$). The interaction plots drawn up between direction and gender, indicate that 6-OHDA did not alter use of the right (non-impaired) limb for the various dosages of 6-OHDA ($F_{(2,24)} = 0.28$, $p = 0.75$). Differences were only detected in terms of the number of steps taken by the left (contralateral) forelimb and thus further analyses were restricted to use made by the left forelimb only. A significant interaction was detected between direction and gender ($F_{(6,90)} = 8.4$, $p < 0.05$). In addition, a significant two-way interaction was revealed for gender and treatment ($F_{(6,90)} = 21.29$, $p < 0.05$). A *post hoc* Bonferroni multiple comparison of the means show that the greatest difference between males and females occurs in the group treated with the higher concentration of 6-OHDA ($p < 0.0001$) (Fig. 4).

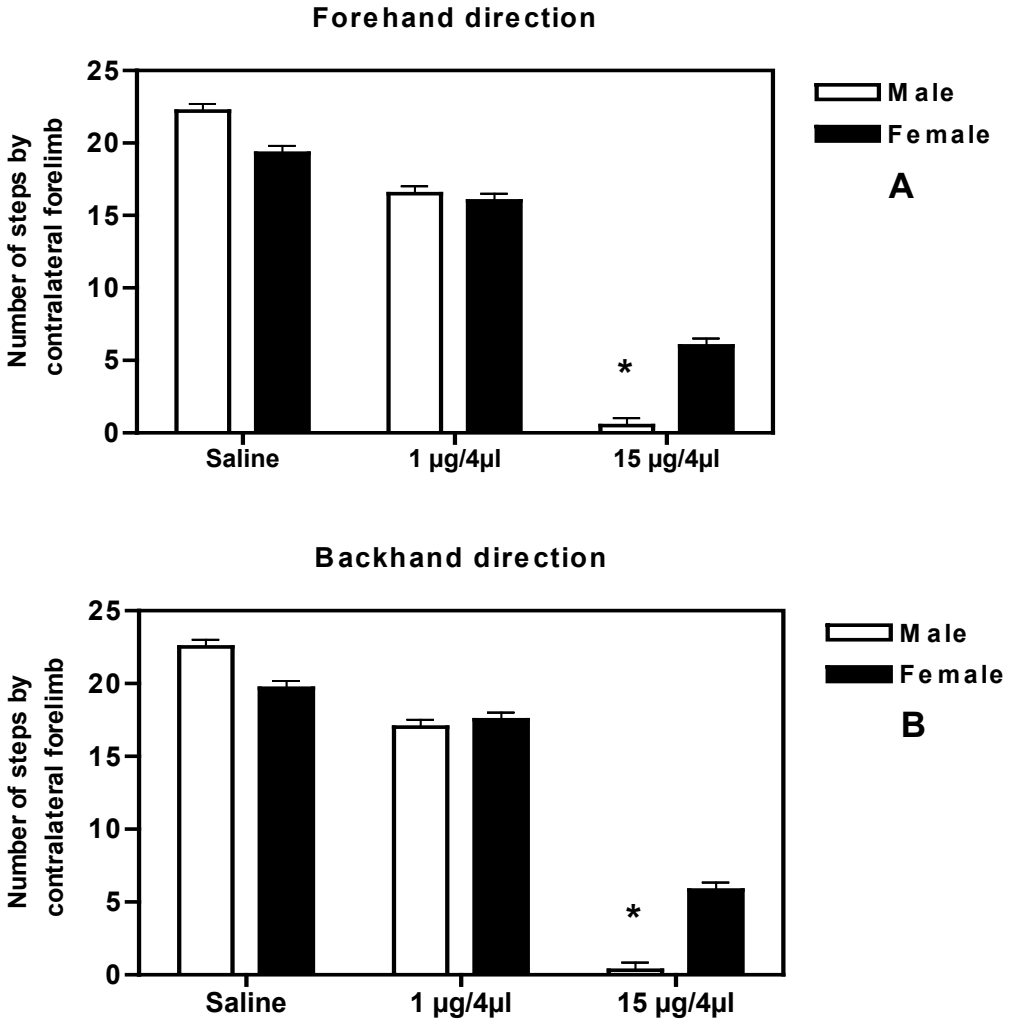

Fig. 4. The mean number of steps taken by the left forelimb in both the forward (A) and backward (B) direction for the adjusting-steps test. A significant difference between the two gender groups exposed to the higher dose of neurotoxin was observed. The ***** symbol signifies a significant difference ($p < 0.0001$) from female rats that had also received the higher dose of 6-OHDA. Data are expressed as mean ± S.E.M. ($n = 6$).

3.3.2. Vibrissae-evoked forelimb placing response

Since a comparable difference was not detected between the different dosage- and gender-groups when stimulating the rats' ipsilateral (right) vibrissae, these data were excluded from the analysis and only the placements made in response to stimulation of the contralateral (left) vibrissae were statistically analyzed. Gender, dosage and the side on which the vibrissae stimulation occurred were the variables considered for analysis. There was a significant interaction between the variables gender and dose ($p < 0.05$). After applying a

Bonferroni correction for multiple comparisons between means and analysing the resultant interaction plots and interaction means, no performance difference was found between the different genders that had either received the low dose of 6-OHDA or saline. However, for the rats that were given higher doses of 6-OHDA, there was significant difference between the two gender groups in the mean number of times when no response was given to stimulation or the number of times that the rats scored zero (see Appendix B, for a complete description of the test) ($p < 0.0001$) (Fig. 5). Impairment was greater in the male rats at the higher dose of 6-OHDA.

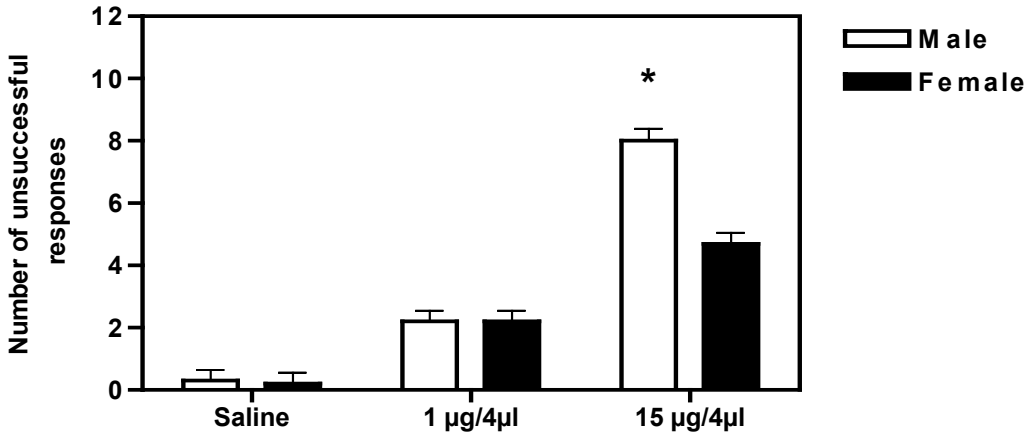

Fig. 5. Results obtained from the vibrissae-evoked forelimb placing test show the number of unsuccessful placing responses made by the forelimb contralateral to the 6-OHDA–infused hemisphere. Data was obtained at 14 days post-operation. The difference between the 2 gender groups administered the higher treatment dosage was statistically significant. The symbol * indicates that the male rats made significantly more unsuccessful responses than female rats ($p < 0.05$) that received the same dose of 6-OHDA. Data represent the mean ± S.E.M. ($n = 6$).

4. DISCUSSION

Gender differences in neural and behavioral indices of brain function in experimental and clinical neurology are widely reported (Li *et al*., 2004; McEwen *et al*., 1975; Milanov *et al*., 2001; Sa & Madeira, 2005). In the current study, we found a significant gender behavioral difference in rats after exposure to a dose of the DA neurotoxin 6-OHDA in tests sensitive to the degree of DA depletion, namely the adjusting-steps and the vibrissae-evoked forelimb placing tests. This finding corresponds to other reports of impaired spontaneous locomotor activity in males compared to females, even when the lesion was not severe and therefore

(Cass *et al*., 2005). For the current study, male rats showed a greater behavioral impairment at 14 days following the infusion of the toxin 6-OHDA at the higher, but not at the lower dose of 6-OHDA, in both the behavioral tests used. In addition, at the higher dose of 6-OHDA, the density of staining for TH in the striatum that had previously been injected with the toxin was reduced in male rats compared to females. These data suggest that the female brains were more significantly protected from DA cell degeneration than the male brains. This finding supports other reports of a greater DA depletion in adult male rats after administering the neurotoxin MPTP (Dluzen & McDermott, 2002; Heller *et al*., 2001). In addition, Murray *et al*. (2003) reported significant gender differences in response to a 1 µg dose of 6-OHDA, with the DA depletion in adult female rats approximately half of that observed in males. However, this difference was lost when higher doses (3 ug and 6 ug) were administered. Other investigators (Becker & Ramirez, 1981; Crowley *et al*., 1978; Fernandez-Ruiz *et al*., 1992; McDermott et al., 1994; Moroz *et al*., 2003) have also shown a sexually dimorphic response to the toxin, with significantly less DA depletion occurring in the female striatum.

The potential mechanisms responsible for the phenomenon remain to be identified, but it is unlikely that a single gender difference in response to 6-OHDA mediates the neural or behavioral effects observed. A surprising finding that emerged from the study was that NGF levels were upregulated in the striatum that had been infused with 6-OHDA, but only in female rats and only at the higher 6-OHDA dose. Several trophic factors are expressed in both the developing and mature SN and striatum (Barroso-Chinea *et al*., 2005; Hynes *et al*., 1994), such as NGF, BDNF, NT-3 and neurotrophin-4/5 (NT-4/5). A decline in their synthesis levels or impaired signal transduction in ageing neurons may contribute to neurodegenerative conditions (Mattson & Magnus, 2006).

Furthermore, since these compounds exert their effects on selective types of neurons they are considered promising therapeutic agents for treating neurodegenerative disorders where the underlying pathological event is the selective degeneration of specific neuronal groups (Garcia de Yebenes *et al*., 2000). Evidence to support this includes research suggesting that sufficient levels of growth factors may be required for maintaining the viability of the remaining DA-producing neurons in pathological conditions, such as PD (Torp *et al*., 2006). Although recent research has predominantly focused on the capacity of other neurotrophic factors, such as GDNF and BDNF, to correct behavioral deficits and protect nigrostriatal neurons against DA neurotoxicity (Cohen *et al*., 2003; Moroz *et al*., 2003; Sun *et al*., 2005), the current experiment focused on NGF instead.

NGF is a neurotrophic agent that is frequently overlooked for its potential therapeutic role in PD. NGF is a neurotrophin on which many sensory and sympathetic neurons depend for their survival during the developmental phase (Yu & Liao, 2000; Zhou & Rush, 1996). Furthermore,

its antioxidant (Salinas *et al*., 2003), anti-apoptotic (Shimoke & Chiba, 2001) and neurorescue properties (Stromberg *et al*., 1990) have been shown to have beneficial effects in neural transplantation studies, with exogenously applied levels that prolonged graft survival. Decreased concentrations of NGF were also detected in PD patients and in experimental parkinsonian rats (Lorigados Pedre *et al*., 2002).

Rats were exposed to the neurotoxin 6-OHDA during the "developmental longevity" phase of their life-cycle when it was less likely that the females could benefit from the potential neuroprotective effects of sex hormones. According to Knoll *et al*. (2000), this period extends from the discontinuation of suckling by the end of the third week of age, until the end of the second postnatal month when sex hormones begin to be detected reliably (Knoll & Miklya, 1995). A characteristic of this time-frame is an enhanced level of catecholaminergic and serotoninergic activity in discrete brain areas (Knoll *et al*., 2000). When sex hormone regulation commences by the end of the rat's second month of life, the activity of noradrenaline, DA and serotonin in the brain is substantially suppressed, suggesting that the "developmental longevity" phase has by then largely ended (Knoll & Miklya, 1995). Several studies support the view that the female brain develops in the absence of estrogens while the development of the male brain is due to the presence of estrogens (MacLusky & Naftolin, 1981; Weisz & Ward, 1980). However, this view has been challenged by those who suggest that the female brain might depend upon the presence of estrogens (Bakker *et al*., 2002; Döhler *et al*., 1984; Fitch & Denenburg, 1998; Toran-Allerand, 1984). Technological advances, such as the A*fp* (A*fp* −/−) mutant mouse model (Gabant, 2002) have provided a tool with which to measure brain estrogen levels (Bakker *et al*., 2006) and to produce findings, which corroborate the original hypothesis (McEwen *et al*., 1975). This classical view of sex-related brain development proposes that the female brain requires protection from estrogens, and its male-typical effects. Therefore, we administered 6-OHDA to rats at postnatal day 35, when their sex hormone levels were likely to be low or undetectable, while the activity of central monoaminergic neurons was suspected to be high. However, it is acknowledged that estrogen may have been decidedly higher at the tissue-collection stage, since the synthesis of AFP, a plasma glycoprotein that shows a high affinity for binding to estrogen (McEwen *et al*., 1975; Raynaud, 1973; Toran-Allerand, 1984) has been shown to decrease rapidly following birth, with only trace amounts detected in adult mice and rats (Andrews *et al*., 1982). However, recent evidence for estrogen-independent sexual dimorphism suggest that, independent of the maculating effects of gonadal secretions, gene expression influences the patterns by which XY and XX brain cells differentiate and function. Behavioral tests, such as a cognitive task of passive avoidance also show striking gender differences, with the female rats that outperformed the males in the successful retention of the task that was independent

of hormonal or surgical treatment. Similar results have been obtained for the corner test, which sensitively detects the degree of damage evoked by experimental stroke (Li *et al*., 2004). Moreover, evidence exists that the testes-determining gene *Sry* is required for normal motor function and TH expression in DA neurons of the male SN (Dluzen & McDermott, 2002; Dluzen & Horstink, 2003). Progesterone, a neurosteroid that is synthesized by the brain in the presence of steroidogenic enzymes, receives far less focus than estrogen in studies concerning the biological basis of gender difference. However, it is known to possess important properties that can enhance the repair of neurodegenerative and traumatic injuries sustained by the CNS. It offers a membrane stabilizing effect that serves to reduce the damage caused by lipid peroxidation. In addition, it may also provide neuroprotection by suppressing neuronal hyperexcitation (Roof & Hall, 2000). It has shown neurorescueing properties in a parkinsonian mouse model, by preventing striatal dopamine depletion caused by MPTP (Cass *et al*., 2005). Nevertheless, not all results have shown such promise. For example, in the absence of estrogen, progesterone did not prevent neuronal loss induced by kainic acid (Azcoitia *et al*., 1999). In addition, in ovariectomized female rats, following experimentally induced middle cerebral artery occlusion, exogenously applied progesterone even exacerbated striatal stroke injury (Murphy *et al*., 2000).

Therefore, it is possible that endogenous progesterone may have provided additional neurocapacity to the animals by the time that the striatal tissue was collected for biochemical analysis. However, as a sex hormone, it would not have been present in the brain by the commencement of the experiment, since this period entailed the “developmental longevity” stage of the rat’s life-cycle (Knoll *et al*., 2000). In summary, very young female rats were better able to resist the neural and behavioral effects of 6-OHDA than male rats of similar age. The data from the current experiment suggest that estrogen-independent gender differences, perhaps together with estrogen-dependent ones, may conceivably contribute to the reduced incidence of PD in women. Behavioral outcome measures correlated with the increased expression of NGF on the hemispheric side infused with the toxin, and with a higher degree of TH expression. However, it can not merely be assumed that a causal relationship exists between the NGF data and all other parameters taken.

The biological basis underlying gender differences in PD are likely to be multi-factorial, and the relative contributions of hormonal, environmental and genetic events throughout the lifespan remain to be determined. Future research endeavors should thoroughly explore all potential candidate mechanisms that may underlie the gender difference detected in the 6-OHDA model of PD. What remains important, however, is that whatever factors are responsible for the females showing better functional outcome and higher levels of TH expression, the current study has shown that the mechanism is functional even from an early

phase of life. This further suggests that gender differences, based on functionally compromised nigrostriatal neurons, can possibly commence at any stage throughout the life-span, and not only during the period that estrogen is available in high concentrations.

5. CONCLUDING REMARKS

In summary, young female rats were better able to resist the neural and behavioral effects of 6-OHDA than male rats of similar age. The data from the current experiment suggest that estrogen-independent gender differences, perhaps together with estrogen-dependent ones, may conceivably contribute to the reduced incidence of PD in women. Behavioral outcome measures correlated with the increased expression of NGF on the hemispheric side infused with the toxin, and with a higher degree of TH expression. However, it can not merely be assumed that a causal relationship exists between the NGF data and all other parameters taken. The biological basis underlying gender differences in PD are likely to be multi-factorial, and the relative contributions made by hormonal, environmental and genetic events throughout the lifespan remain to be determined. Future research endeavours should thoroughly explore all potential candidate mechanisms that may underlie the gender difference detected in the 6-OHDA model of PD. What remains important, however, is that whatever factors are responsible for the females showing better functional outcome and higher levels of TH expression, the current study has shown that the mechanism is functional, even from an early phase of life. This further suggests that gender differences, resting on the basis of functionally compromised nigrostriatal neurons, can possibly commence at any stage throughout the life-span and not only during the period that estrogen is available in high concentrations.

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CHAPTER 4

A COMPARATIVE EVALUATION OF BEHAVIOR, DOPAMINE AND DOPAMINE'S METABOLITES AFTER STRIATUM VS. MEDIAL FOREBRAIN BUNDLE SITE OF 6-OHDA ADMINISTRATION IN JUVENILE RATS

1. INTRODUCTION

PD is the second most common neurodegenerative disease (de Lau & Breteler, 2006), affecting nearly 2% of the population over the age of 65 (Haugarvoll & Wszolek, 2006). Although certain monogenetic mutations may be responsible for the manifestation of the disease, these account for only 5-10% of all cases and the majority show idiopathic and sporadic disease onset (Bonifati, 2005; Dauer & Przedborski, 2003). Akinesia and rigidity comprise the characteristic clinical features of the disease (Schuller & Marshall, 2000), caused by extensive degeneration of the nigrostriatal pathway. This fiber-bundle connects two nucleic regions, the SN from where the axons originate from, and the striatum, to where they project towards and eventually terminates within specific dendritic regions of the medium spiny projection neurons located in the dorsal striatum (Moore *et al*., 1971; Fallon *et al*., 1978). Both neuronal structures fulfill a critical role in voluntary motor control (Wooten, 1997; Schober, 2004) through the axonal relay of the neurotransmitter dopamine (DA) via the medial forebrain bundle (MFB). The striatum also provides an anatomical substrate at which cholinergic, glutamatergic and DA-ergic interactions occur (Bouyer *et al*., 1984) and acts as the primary input nucleus to the subcortical forebrain system, collectively known as the basal ganglia (Schuller & Marshall, 2000) that comprise of a group of structures involved in processing highly associated cortical information. These functions include a contribution made to the complex process of planning and selecting appropriate movements and behavior within a given context (Wise *et al*., 1996; Mink, 1996; Redgrave *et al*., 1999). Therefore, the deregulated movement patterns (dyskinesia) associated with PD may be interpreted as the mismatched selection of motor responses to contextually derived sensory stimuli (Leblois *et al*., 2006).

No existing animal model completely simulates all the pathological features relating to PD. However, the ability of certain neurotoxins, such as 6-OHDA to mimic certain clinical features of the human disease in rats has greatly improved our understanding concerning the disease's pathophysiology and has contributed towards developing novel therapies, including L-DOPA that remains the "golden standard" for symptomatically managing the disease (Betarbet *et al*., 2002). The toxin induces the selective destruction of catecholamine neurons and damages the mitochondrial electron transport system (Olanow, 1993) to lower the supply levels of available cellular energy (Sauer & Oertel, 1994), while it has also been shown to augment glutamate release (Halliwell, 1992).

A direct, unilateral injection of 6-OHDA into the DA-ergic pathway, targeting the MFB, in which case DA originates from the A9 cell group located in the SNpc (Bobillier *et al*., 1975), is a common method for administering a 6-OHDA lesion. The release of a toxic insult near the DA-ergic neuronal cell bodies result in a near-immediate and complete loss of DA-ergic neurons (Schwarting & Huston, 1996), instead of the gradual neuronal death observed in the clinical entity (Finkelstein *et al*., 2000; Yuan *et al*., 2004), and is therefore considered representative of the late end-stages of the disease. An alternative approach involves injecting the toxin directly into the striatum (Berger *et al*., 1991; Ichitani *et al*., 1994; Sauer & Oertel, 1994; Przedborski *et al*., 1995; Lee *et al*., 1996), thereby directly damaging the DA-ergic axonal endpoints, resulting in the slowly evolving death of a subpopulation of SN cells by means of a retrograde mechanism (Sauer & Oertel, 1994). The model is considered to effectively mimic the slow cellular demise that characterizes disease development (Shimohama *et al*., 2003), although the SN nuclei cells degenerate at a far slower pace in the clinical entity (Di Paola & Uitti, 2000). An additional advantage of the striatum-model is that it allows for a relatively long window-period for cellular demise to occur, during which the ability of potential therapeutic agents to rescue toxically impaired cells can be tested (Fleming *et al*., 2005).

Although both lesion-types evoke a loss of DA that results in reproducible and quantifiable contralateral motor deficits (Lee *et al*., 1996; Cohen *et al*., 2003), few studies have directly compared the effects of the two lesion-types, in terms of the levels of DA and its primary metabolites remaining in the striatal tissue, as well as the motor-behavioral profile they evoke. In addition, the vast majority of studies have made use of older animal subjects while limited studies have focused on the effects left on the developing CNS. For the present study, we used a unilateral rat model of neurodegeneration, by injecting the toxin 6-OHDA into the MFB to induce a lesion in the nigrostriatal DA-ergic (NSDA) pathway of very young rats (35 days old). The behavioral and neurochemical effects were compared to a direct unilateral injection into the striatum, in rats of a similar age and gender-type (male). We employed a motor-function ('cylinder') test that is sensitive to the degree of lesion-induced disability and possible recovery from it. In addition, the extent of the lesion produced at both sites was quantified using HPLC coupled to an electrochemical detector (HPLC-EC), to determine the amount of striatal DA and DA metabolites (3, 4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) remaining in the post-mortem brain.

2. MATERIALS AND METHODS

2.1. Animals

Sixteen male Sprague-Dawley rats were used for this study. The rats were randomly divided into one of two groups: those that had either received a partial lesion (n = 8) by injecting the 6-OHDA unilaterally into the striatum, or rats that had sustained a complete lesion (n = 8), by injecting the toxin into the MFB. All animals were kept in an AAALAC accredited animal holding facility

(University of Stellenbosch) and housed in groups of four, with housing consisting of clear 40 x 25 x 20 cm Plexiglas cages containing sawdust, with rat pellets and tap water provided *ad libitum*. A 12 hr light/dark cycle (lights on from 07h00 to 19h00) was maintained within the animal holding facility. All experimental treatments that were used conformed to international ethical standards and the protocol has been approved by the local (University of Stellenbosch) Committee for Experiment Animal Research (project number: N05/04/004).

2.2. Preoperative & Surgical Procedures for the Infusion of 6-hydroxydopamine into the MFB or striatum

For stereotaxic lesioning, the MFB and striatal coördinates were confirmed by lesioning four/lesion-type 35 day-old (75-85g) rats with India ink. Following the injection the rats were killed by decapitation and the brains placed in paraformaldehyde for 48 h, after which the tissue was cut with a cryostat at a thickness of 30 µm. The sections were then mounted onto clear glass slides and examined under a Nikon TE2000-E microscope (using a phase 4 resolution) to determine the location of the tip of the needle, while referring to a stereotaxic atlas of the rat brain (Paxinos & Watson, 1986). The original dorso-ventral coordinates were adjusted and the procedure was repeated until the correct placement of the needle could be confirmed by two investigators. Bregma and Lambda were used to calculate the coordinates used for the MFB and the striatum injection, which were calculated from the dura mater, in order to cancel the individual variability observed in skull thickness. For the MFB lesion the coordinates were: 2.5 mm anteroposteriorly (AP), 1.8 mm mediolaterally (ML) and 7.4 mm dorsoventrally (DV), while for the striatum injection they were AP:0.57, ML:2.73 and DV:5.57.

Animals were randomly assigned to either the MFB or the striatum-injection. At PND 35, 6-OHDA was infused unilaterally (right hemisphere) into either the MFB or the striatum. Thirty minutes prior to receiving the anaesthetic injection and immediately following surgery, the rats from both groups were pretreated with Desipramine HCl (Sigma, South Africa, 20 mg/kg i.p.), dissolved in sterile saline (NaCl 0.9%). The drug functions as a noradrenaline-reuptake inhibitor to protect noradrenergic neurons by preventing 6-OHDA from entering the cells (Cohen *et al*., 2003; Espejo & Miñano, 2001). The rats were anaesthetized using a combination of ketamine hydrochloride (Anaket-V®, Bayer Healthcare, South Africa) and medetomidine hydrochloride (Domitor®, Pfizer, South Africa) (0.1 ml/100g, i.p.). The head of the animal was shaved and it was placed in a stereotaxic apparatus (David Kopf instruments, CA, USA). The skull was exposed and the cranium leveled in the horizontal plane. A burr hole was drilled in the skull using the predetermined striatal or MFB coordinates. Infusions of 6-OHDA (Sigma, USA; 12µg) dissolved in 4µl of 0.1% L-ascorbic acid which in turn was dissolved in 0.9% sterile saline was administered. The toxin was delivered

through the burr hole by using a Hamilton 10 µl syringe with a 22-gauge needle attached, driven by a 341-A model syringe pump (Sage Instruments, USA) at a rate of 1µl/min for 4 min. The cannula remained *in situ* for an additional 5 min following the administration to prevent reflux of the toxin along the injection tract, before slowly retracting it from the brain tissue and skull. The hole drilled in the skull was filled with sterilized oxidized cellulose (Ethicon, U.K.), the wound sutured and covered with a layer of iodine. The animal was kept under a heating-lamp until full recovery from the anesthesia. Rats were then returned to their cages and their food and water in-take as well as general behaviour was monitored daily to ensure their full recovery.

2.3. General behavioral testing procedures

Animals were tested for behavioural changes at 28 days post 6-OHDA-injection. Testing consisted of the *forelimb-use asymmetry* ('cylinder') test. All testing took place between 10h00 and 12h00, in a quiet, dimmed room dedicated to animal testing.

2.3.1. Forelimb-use asymmetry ('cylinder') test

A lesion sustained on one hemispheric side manifests in spontaneous behavioral alterations with these measures serving as litmus tests for confirming lesion size (Schwarting *et al*., 1991; Sindhu *et al*., 2005). Twenty-eight days following the surgery, drug-free behavioral testing was performed, since it reflects a more natural response to the effects of the lesion (Roghani *et al*., 2002). The forelimb-use asymmetry test ('cylinder') was used, since asymmetry scores in forelimb usage during the vertical-lateral exploration of the inner-walls of a cylindrical chamber has been shown to correlate with the extent of nigrostriatal terminal loss resulting from the toxin's infusion (Schallert & Woodlee, 2005; Schallert, 2000). For this test, rats were individually placed in an upright transparent Plexiglas cylinder, with the diameters measuring at 19 cm across and 24.4 cm in height. The walls were high enough to prevent the animal from reaching the top edge when standing on its hind limbs and were wide enough to permit a comfortable 2 cm space between the tip of its snout and the base of its tail when all four paws were placed on the ground. The floor of the container consisted of a transparent sheet portraying four quarter-of-a-circle sections all equal in size.

The parameters used for quantifying the degree of forelimb-use asymmetry were: (1) the independent use of a single forelimb (left vs. right) for exploring the inside surface of the cylinder, while the other limb remained stationary, but in constant contact with the inner-surface of the cylinder wall; and (2) the amount of stereotypic rotations the animal made towards (ipsilateral) and away (contralateral) from the lesioned (right) side were recorded. A single rotation was counted if the animal made a complete continuous 180° turn in either direction, correlating with the rat moving through at least two of the quadrants marked on the cylinder's floor. For parameter (1) the limb-use score was calculated using the number of times that the ispsilateral (left) and contralateral (right)

had been placed on the inner-wall surface divided by the total number of forelimb placements (ipsilateral + contralateral + both) and expressing this as a percentage by multiplying the value with 100 (Connor *et al*., 1999). For parameter (2) a single score was calculated by subtracting the amount of contralateral turns made from the ispsilateral ones. Other counts included were the amount of steps taken by each of the backlimbs (2), and the amount of times that the animals made a full rear against the inner wall of the container, as a measure of gross motor function.

The animals were tested during the light phase (between 10h00 and 12h00) with each test lasting 5 min in total, following which the rats were immediately returned to their home cage. A video recorder with slow-motion and frame-by-frame capabilities mounted on a tripod at an angle and underneath the cylinder, allowed full-view of the animal's activities, even when the rat turned away from the camera. The rat's behavior was analyzed from the video recording by a rater blind to the experimental condition of the animals. In cases where it was unclear whether the limb had been placed independently or simultaneously with the other, it was neglected from the final score.

2.4. HPLC-EC analysis for DA, DOPAC and HVA

Following the last behavioral assessment, all animals were killed by decapitation and the striatum tissue was collected for analyzing the DA and DA metabolite content level. The brains were rapidly removed from the skull and the total striatum of both hemispheres were dissected out on an ice-cooled dissection slab by cutting coronal sections at the level of the infundibular stem to form a forebrain block containing the striatum. The forebrain was bisected along the midline and cortical tissue for each hemisphere was peeled back to expose the striatum, which was dissected along the boundary of the corpus callosum. The striata were snapped frozen in liquid nitrogen (-198 ºC) until further analysis. DA and DA-metabolites content levels were determined by the same HPLC-EC method as was previously described by Harvey *et al.* (2006). The chromatographic system consisted of a GBC LC 1120 HPLC Isocratic pump that contained an inlet filter and a Rheodyne 7725i injection valve comprising of a 50 µl loop for introducing samples into the system. For the separation step, a 5µm C_{18} Phenomenex Luna reverse phase analytical column (150 × 4.6 mm) was connected to a GBC LC 1260 Electrochemical Detector (+0.6 V) as well as a Spectra-Physics SP4290 integrator. The mobile phase consisted of 0.1 M sodium formate buffer, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM sodium heptane sulphonic acid, 6% v/v methanol and 4% v/v acetonitrile. Milli-Q water was used to make the solution up to 1000 ml. The pH was set at three by adding 6 ml of orthophosphoric acid (85%). The composition was degassed twice prior to use. The flow rate of the mobile phase running through the system was set at 1.2 ml/min. The temperature remained ambient throughout. On the day of analysis the samples were allowed to thaw and the weight of each sample recorded. A total volume of 1 ml 0.1 M perchloric acid solution was added to each tube, sonicated and then placed on ice for 20 min, after which the tissue was

centrifuged (20 min, 4˚C, 24 000 × g, Sorval Discovery 9056 ultracentrifuge). Following the withdrawal of 200 µl aliquot of supernatant, 20 µl of the internal standard solution (isoprenaline HCL) was added and the solution mixed. A sample of 50 µl was injected into the column for content analysis. Standard DA, DOPAC and HVA solutions were prepared in a vehicle of 0.5 mM sodium metabisulphite and 0.3 mM Na_2EDTA, diluted with perchloric acid (0.1 M). The monoamine standards ranged between 10 ng/ml – 200 ng/ml and linear standard curves were found in this particular range. DA, DOPAC and HVA were quantified by comparing the area under the curve (AUC) of the sample/AUC of the internal standard (isoprenaline) to the monoamine standard, utilizing the linear equation obtained from the standard curve. ChemStation Rev A 08.03 software was used for data acquisition purposes.

2.5. Statistical analysis

Comparisons for differences in means and variance were statistically assessed by means of a 2-way Analysis of Variance (ANOVA) using a 2 (site of injection) × 2 (the limb side used by the animal) × 2 (behavioral assessment time-points) factorial design. This was followed by a *post hoc* multiple comparison *t*-test (Bonferroni) when appropriate. For an analysis of the number of rearings made by the two groups during the 'cylinder' test, a parametric Student's t-test was used for analyzing the data. Differences were regarded as significant when $p < 0.05$. The statistical analysis of the data was performed using the Prism® statistical analysis software program (GraphPad Software, version 4.02, USA). All data are expressed as mean ± the standard error of the mean (S.E.M.)

3. RESULTS

3.1. Independent use of the forelimbs

An ANOVA analysis of the data obtained from the "cylinder" task revealed no significant 2-way interactions ($F = 0.03$, $df = 1$, $p = 0.86$) between the factors site of administration (MVB vs. striatum) and independent limb use for exploring the inside of the cylinder (ispsilateral vs. contralateral). *Post hoc* comparisons revealed a significant effect for the preferential use of a single forelimb ($F = 40.12$, $df = 1$, $p < 0.0001$), but not for site of administration ($F = 0.80$, $df = 1$, $p = 0.38$). Fig. 1 shows the striatal-lesioned group's performance compared to their MFB counterpart. The figure shows a decrease in the percentage contralateral limb use, compared to the ipsilateral limb use in both groups.

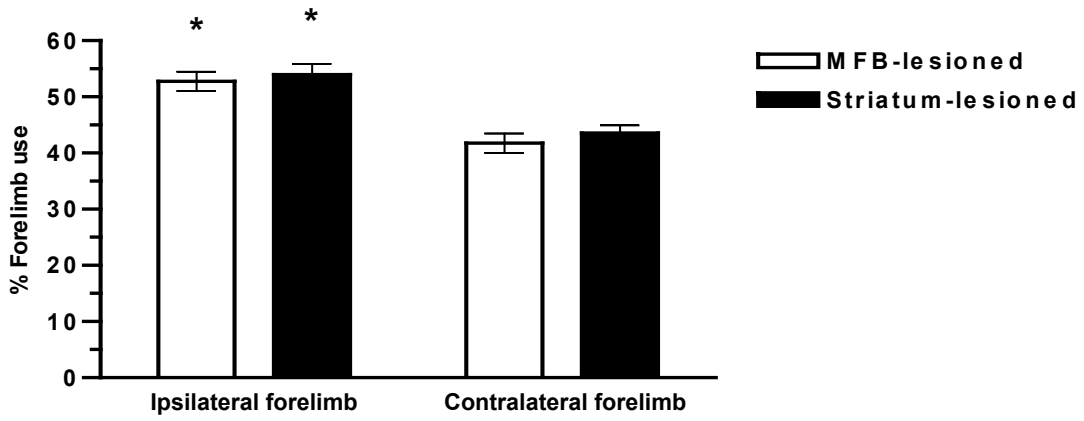

Fig. 1. Independent placing responses made by the ispsilateral and the contralateral forelimb during the 'cylinder' task. For both groups, the asterisk (*) indicates a significant (p < 0.05) difference compared to the contralateral limb.

3.2. Spontaneous rotation

A 2-way ANOVA was performed to determine for the effect of the factor site of administration (MFB vs. striatum) on the dependent variables direction of the turns made (ipsiversive vs. contraversive) and the number of 180° rotations in either direction, followed by a Bonferroni multiple comparison *post hoc* analysis. The results revealed no overall significant interaction between the factors (F = 2.4, df = 1, p = 0.13). A significant overall effect was detected for the direction of turning (F = 10.1, df = 1, p = 0.004), but not for the site of administration (F = 0.003, df = 1, p = 0.96). Although both groups tended to rotate more in a contraversive than in an ipsiversive direction, only the striatal-lesioned group showed a statistically significant preference for rotating away from the lesioned hemisphere (Fig. 2).

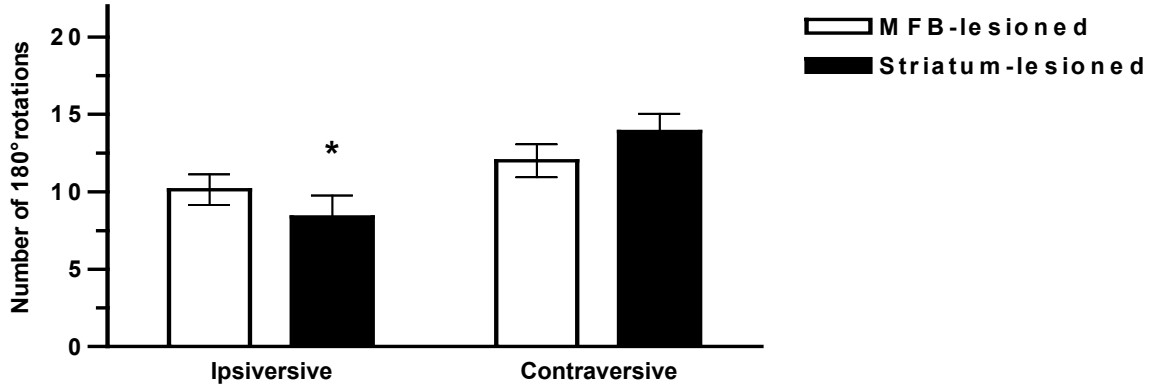

Fig. 2. The number of spontaneously induced continuous 180° rotations towards (ipsiversive) and away (contraversive) from the 6-OHDA lesioned hemisphere. The * indicates a significant (p < 0.05) difference when comparing the number of contraversive to ipsiversive turns, made by the same animal group.

3.3. Number of steps taken by the hindlimbs

No significant overall interaction was detected for this parameter allowing for main effects to be interpreted. There were no significant differences between the limb side used (F = 2.47, df = 1, p = 0.13). However, a significant effect was detected for the site of administration (F = 4.25, df = 1, p = 0.049). As shown in Fig. 2, n both groups, use of the contralateral backlimb did not reach significance compared to use made of the ispsilateral backlimb (Fig. 3).

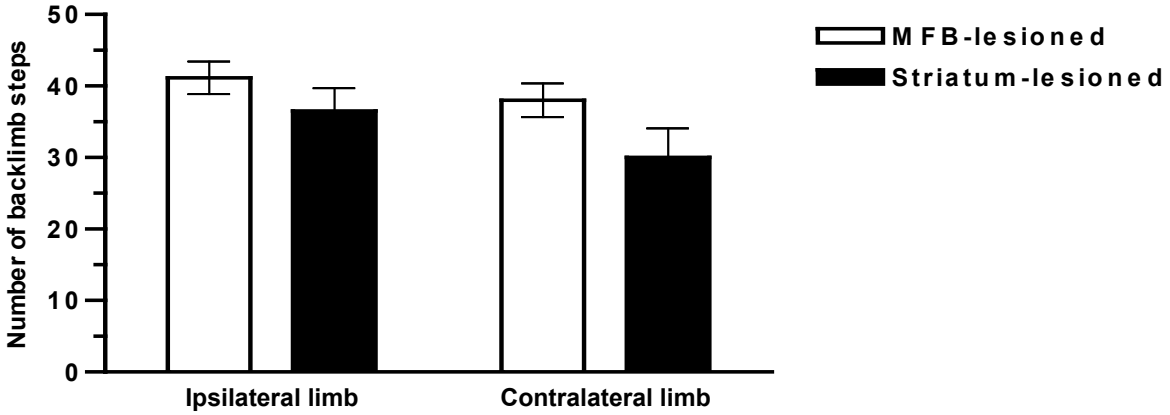

Fig. 3. The number of stepping movements by the hindlimbs following a 6-OHDA lesion. No significant difference was observed between use made by the ipsilateral and the contralateral backlimb, in either group (p > 0.05).

3.4. Rearing frequency

There was a significant difference detected between the amount of full-body rearing movements made by the MFB and the striatum group (F = 4.25, df = 7, p = 0.04) (Fig. 4). A similar observation, but in older adult rats, had previously been reported by other investigators of a significant increase in locomotion and rearing (Castañeda *et al*., 1990) depending on the size of the lesion. The results showed that the size of this parameter may depend on the site at which the toxin was administered to juvenile rats, since the MFB-lesioned rats displayed significantly reduced rearing movements compared to the striatum-lesioned group (Fig. 4).

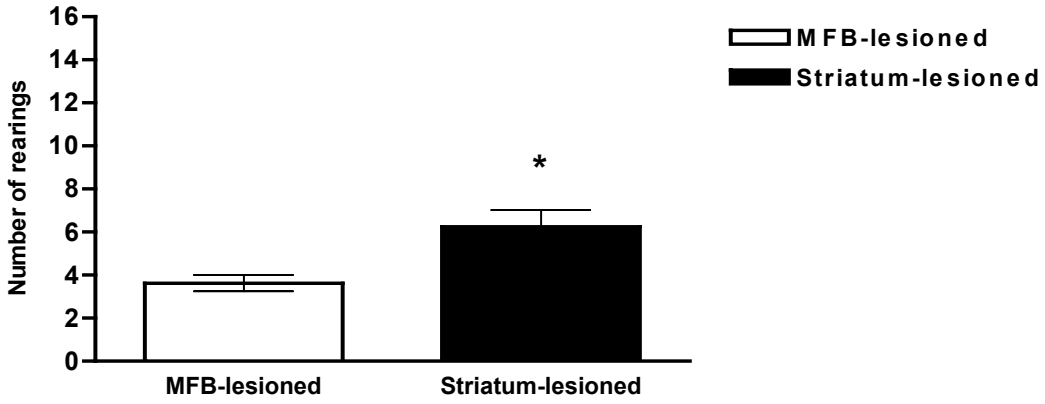

Fig. 4. The degree of rearing movements recorded at 28 days following the 6-OHDA lesion. The * indicates a significant ($p < 0.05$) difference when compared to the MFB-lesioned group.

3.5. HPLC-EC results

A 2-way factorial ANOVA with a 2 (site of toxin administration) × 2 (hemispheric side from which the striatum was dissected) design was used for analyzing the data obtained from the HPLC-EC analysis performed on the striatum samples. For the statistical analysis of the DA values, no significant interaction was found for the site of administration ($F = 0.01$, $df = 1$, $p = 0.92$) or striatum side ($F = 2.03$, $df = 1$, $p = 0.17$), while no significant overall effect was found between DA levels, site and hemispheric side ($F = 2.18$, $df = 1$, $p = 0.1.5$). Fig. 5A shows the results of these analyses, in which the DA levels in the MFB group had been unaltered by the lesion, while a DA upregulation was observed on the lesioned side in the striatum group.

For the DOPAC levels, a significant effect was detected for the factor site of administration ($F = 9.01$, $df = 1$, $p = 0.006$), but not for the factor striatum side ($F = 0.03$, $df = 1$, $p = 0.85$). A significant overall effect was found between the factors site of administration and striatum side ($F = 15.48$, $df = 1$, $p = 0.0005$). Fig. 5B shows an upregulated level of DOPAC on the lesioned side for the striatal-infused animals, while the opposite holds true for the MFB-infused rats that show a downregulation on the lesioned side compared to the unlesioned intact side.

The HVA values showed no overall significant interaction ($F = 2.76$, $df = 1$, $p = 0.12$) with the site of administration. However, the analysis revealed a significant effect for lesioned vs. unlesioned hemispheric side in the striatal lesioned group ($F = 11.62$, $df = 1$, $p = 0.002$). Fig. 5C shows these results, with a significant difference indicated between the DOPAC levels of the MFB-infused and the striatum-lesioned rats. A difference was detected in both hemispheres.

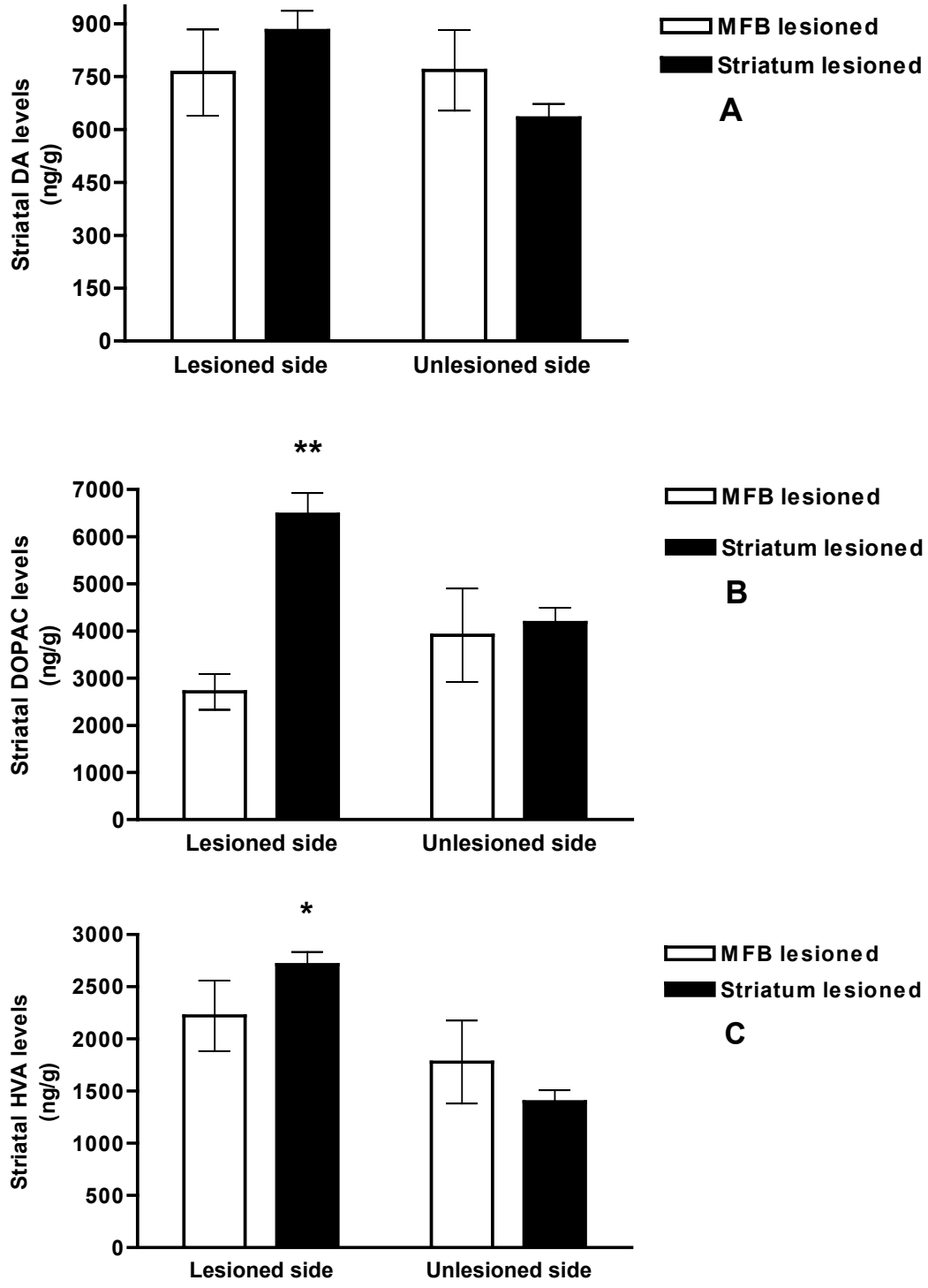

Fig. 5. Results of the HPLC-EC analysis performed on striatal tissue, showing DA (A), DOPAC (B), and HVA (C) concentrations. When comparing the MFB lesioned animals to the striatal lesioned ones, the asterisks indicates statistical significance (*$p < 0.05$, **$p < 0.001$).

An analysis of the DOPAC/DA ratio revealed no significance for striatum side (F = 3.68, df = 1, p = 0.07), but revealed significance for site of administration (f = 36.97, df = 1, p < 0.001), as well as an overall significant interaction effect (f = 16.40, df = 1, p < 0.001). These results are shown in Fig. 6 with an upregulation of DA metabolism seen on the lesioned side compared the unlesioned side in the striatum-infused rats, but the opposite effect seen in the MFB-lesioned group.

The HVA/DA ratio was significant for the striatum side (f = 13.32, df = 1, p = 0.001), but not for the site of administration (f = 0.002, df = 1, p = 0.96). No significant interaction effect was revealed (f = 0.74, df = 1, p = 0.4). In Fig. 6b, the upregulated metabolic effect is seen on the lesioned side compared to the unlesioned side in both groups of animals.

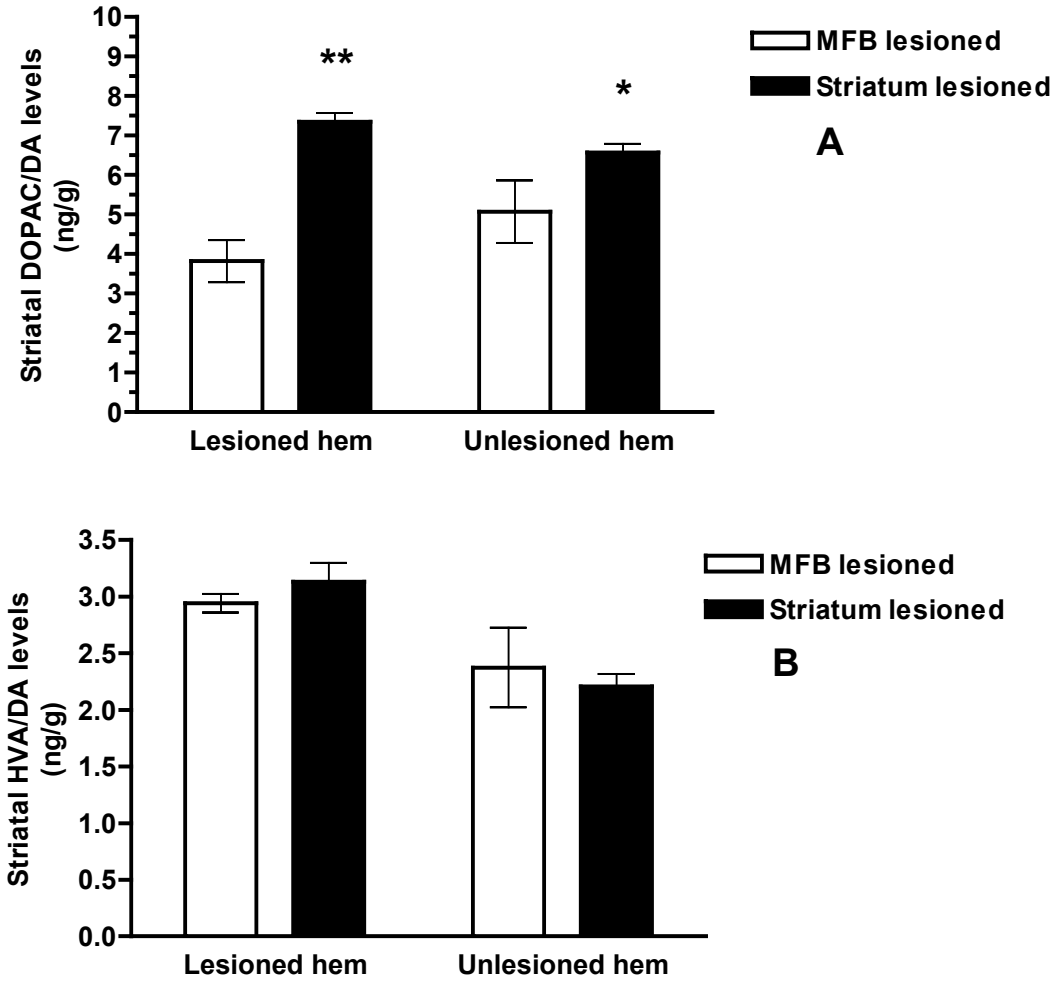

Fig. 6. The DA turnover rate representing DA metabolism in the striatum (Altar, 1987b), and expressed as DOPAC/DA (A) and HVA/DA (B). The asterisks indicate a significant comparison in relation to the MFB lesioned group (*p < 0.05, **p < 0.001).

4. DISCUSSION

Here a parkinsonian animal model was created by centrally administering 6-OHDA, in order to provide evidence for anatomically distinct toxin susceptibility directly at the level of the NSDA neurons in the developing CNS. Lesions were made at two different neuro-anatomical locations within the pathway to determine for behavioral, striatal DA and DA metabolite content differences. The results show that the degree of motor deficits, which were demonstrated by using the 'cylinder' test, a motor-function test that is sensitive to the degree of DA loss, depending on whether the destruction of the NSDA pathway took place at the level of the cell body or nerve terminals. The significant differences in functional impairment between the groups suggest for a different modulation of motor behavior by these different brain regions, possibly due to complex neuromodular organization and inter-linkages.

Post-mortem brain catecholamine levels provide a good index of the density of DA-ergic innervations in rats following recovery from a unilateral 6-OHDA lesion (Ranje & Ungerstedt, 1977; Onn *et al*., 1986; Altar *et al*., 1987b). In the current experiment, neurochemical analysis using HPLC-EC revealed a surprising compensatory DA effect in the lesioned hemispheric side to indicate that the nature and/or sensitivity of the processes whereby 6-OHDA transmits its neuron-damaging effects to the level of the cell body and the striatum may induce different levels of degeneration between the mature and the developing CNS. Although the degeneration of nigrostriatal DA-ergic fibers compiles the hallmark of idiopathic PD (Kish *et al*., 1988), it is debatable what critical threshold should be reached before symptoms express clinically. The adult nigrostriatal system seems to be able to endure substantial DA deficits, the degree of which has not yet been fully described. This inherent reserve capacity might explain why parkinsonian features seldom manifest before the progressive decline of at least 80% of the DA-ergic neurons located in the SNpc, with the slow progression implying that compensatory mechanisms are likely to be in place (Finkelstein *et al*., 2000).

In the experimental lesioning of the nigrostriatal DA system of rats, it's complete (>95%) destruction results in severe motor, sensory, and cognitive deficits which animals fail to recover from (Stricker & Zigmond, 1976; Marshall, 1985; Robinson *et al*., 1990). In cases where the loss of DA-ergic cells exceed the 95% limit, compensatory mechanisms seem to fail with dialysate DA levels decreasing dramatically, even during amphetamine-induced DA release (Robinson & Whishaw, 1988; Castañeda *et al*., 1990). However, certain reports have indicated that even following striatal DA depletion in excess of 95%, a return to basal dialysate DA levels may still occur (Jonkers *et al*., 2000; Bergquist *et al*., 2003).

On the other hand, considerable sparing of function has frequently been observed following a (<80%) depletion of striatal DA (Stricker & Zigmond, 1974). Animals that had sustained partial depletions, comprising of an 80-95% striatal DA loss, initially suffer from severe behavioral deficits, but eventually regain many of their previously-held abilities (Zigmond & Stricker, 1973; Marshall *et*

al., 1974; Marshall, 1979), while dialysate DA levels are seemingly maintained in the striatum. However, this only seems to be the case as long as the level of neurodegeneration does not exceed 80% (Sarre *et al*., 2004). Further evidence for a positive correlation between the magnitude of behavioral dysfunction and DA levels remaining in striatal tissue was provided by human post-mortem studies (Lees *et al*., 1985). In addition, since PD is characterized by the slowly progressive demise of DA-ergic neurons in the SNpc, symptoms only appear after most neurons have been lost (usually >80%). Such a slow course of degeneration may imply that compensatory mechanisms are in place serving to reduce the speed of decline (Finkelstein *et al*., 2000). Taken together, these observations highlight the general consensus that although the NSDA DA system is necessary for maintaining normal motor-function, a mere 10-20% of the DA input to the striatum seems to be required for functioning relatively intact (Robinson *et al*., 1994a). However, considerable individual variation concerning the magnitude of decline, the ability to maintain extracellular DA levels, and in the rate and extent of behavioral recovery contribute towards the lack of consensus on what constitutes the "critical depletion zone" (Marshall, 1979; Kozlowski & Marshall, 1981; Stricker & Zigmond, 1986).

The concept of resistant DA cells gained additional experimental support from reports of similar TH-immunoreactive fiber densities and DA neuron loss between animals lesioned either uni- or bilaterally with 6-OHDA (Roedter *et al*., 2001). Furthermore, following the systemic administration of the DA agonist *d*-Amphetamine, Robinson & Whishaw (1988) and Castañeda *et al.* (1990) reported that DA released on demand in the toxin-lesioned striatum compares favorably to the DA levels detected in the intact striatum. In the current experiment, HPLC analysis of striatal tissue revealed that the MFB lesion had no effect on extracellular DA levels, since the levels compared favorably to those detected on the unlesioned side.

However, in the striatum-lesioned rats the DA in the unlesioned left hemisphere exceeded that of the lesioned side. It may be possible that release from DA terminals were increased in the remaining neurons via increased mesolimbic pathway afferents and/or by decreasing the number of synaptic DA reuptake sites and/or D_4 receptors (Ikegami *et al.*, 2006). The DA neurons responsible for this DA pool may be located in a few remaining striatal terminals that have either undergone partial degeneration or had been spared altogether (Robinson *et al.*, 1994b). However, if the terminals had been partially damaged by the toxin, they are likely to be isolated from their cell bodies, thereby being prevented from receiving stimulatory action potentials (Mintz *et al*., 1986).

Little consensus currently exist regarding the nature of DA-release following a 6-OHDA lesion to the nigrostriatal pathway, where a multitude of adaptations presumably interact to maintain and "normalize" extracellular striatal DA levels (Castañeda *et al*., 1990). The precise role played by presynaptic vs. postsynaptic contributions towards compensatory neuro-adaptations and the animal's eventual functional recovery remains unclear. Some previous studies suggest for

presynaptic adaptations, such as compensatory increases in DA biosynthesis and the rate of release by DA neurons surviving the 6-OHDA insult (Marshall, 1984; Robinson & Whishaw, 1988; Castañeda *et al*., 1991). Furthermore, reports of lowered DA metabolism, an increase in DA's ability to diffuse further (Stricker & Zigmond, 1976; Robertson & Robertson, 1989; Zigmond & Stricker, 1973) and the fractional release of DA in the remaining DA terminals suggest that the lesion may provoke "silent" cells to become active in the wake of the toxic onslaught (Hollerman & Grace, 1988). In addition, some authors have observed vigorous axonal sprouting in the terminal arbors as well as increased axonal varicosity size in lesioned animals, suggesting that substantial sprouting of new DA-ergic synapses may be the main post-synaptic compensatory attempt (Finkelstein *et al*., 2000).

The compensatory effect in DA levels observed in the current experiment may be due to the fact that the toxic insult was delivered to young CNS system, that may have a superior capacity for plasticity over an adult one. The reason for this remains obscure, but it has been suggested that a 6-OHDA injection in neonatal rats cause norepinephrine depletion in the frontal cortex to trigger a compensatory increase in DA, serotonin (5-HT), and met-enkephalins correlated by an increased density of tyrosine hydroxylase and 5-HT-positive axons (Germani *et al*., 1995). The bilateral administration of 6-OHDA often causes severely impairs an animal's ability to feed, drink, and move, to often leads to their death. However, for neonatal rats receiving similar treatment, survival is common (Brot *et al*., 2002).

However, it may be that the normalization of extracellular DA, whether this occurs in the developing or the mature CNS, may not be sufficient to account for functional recovery. In the 'cylinder' test, the remaining DA pool appeared not to be adequately mobilized upon functional demand, and asymmetric performances were observed in a number of behavioral parameters. Although presynaptic compensatory changes maintain normal basal extracellular DA levels in the remaining DA terminals (Parsons *et al*., 1991) and reach their optimal performance within 3 days following a unilateral 6-OHDA lesion (Altar *et al*., 1987a), this fails to explain for the protracted time-course of behavioral recovery following a 6-OHDA lesion to the NSDA pathway.

The MFB group showed significantly less locomotion over that of the striatal-lesioned rats, as was measured by the amount of rearing movements displayed during the 'cylinder' task. This enhanced behavioral response seen in one group compared to the other may be due to normal extracellular levels of DA acting on a supersensitive receptor population in the one, but not in the other group.

At 28 days following the lesion, both the groups turned predominantly away from the lesioned side, thus in a contraversive direction, although the degree did not reach statistical significance. By first observations the tendency to turn contraversively directly opposes the fundamental tenet underlying the rotational behavior model, which states that animals tend to rotate *away* from the

more active striatum (Ungerstedt, 1971a), defined as the hemisphere that releases the greatest amount of DA into intersynaptic spaces. In the current experiment, the striatum animals tended to turn away from the intact striatum and what was presumably the hemisphere of greatest DA-release. The explanation for this might be similar to that proposed for a similar behavioral pattern seen during ligand-induced rotational assessment (Robinson *et al*., 1994b). Although DA-release may be attenuated on the lesioned side, the DA released by DA-ergic neurons within the lesioned hemisphere could interact with supersensitive DA receptors to bring about a greater degree of postsynaptic activation on the lesioned side. In contrast, DA acts upon normosensitive neurons within the intact side (Ungerstedt, 1971b; Costall *et al*., 1976; Staunton *et al*., 1981; Neve *et al*., 1982). A report that even a mild increase in extracellular DA, by correcting a functionally defective adrenal graft, restored impaired symmetry in striatal DA binding to D_2 receptors. Also a tonic increase in synaptic DA was able to down-regulate DA receptors (Curran *et al*., 1993).

Taken together, our results argue that a combination of compensatory mechanisms and various DA sources resulted in sustaining tissue DA levels in the adult brain following a toxic insult to the DA-ergic neurons during the neonatal period. For both groups of rats there were definite signs of compensatory mechanisms at work reflected in the HPLC-EC results, while variations were observed during the behavioral tests. Overall, the behavioral results indicate that the interdependent regulation of the 2 nigrostriatal systems may provide some compensatory support for the function and behavioral performance of the lesioned side via the normal unlesioned side. It should be noted that steady-state striatal DA content represents a complex balance between synthesis, release, reuptake and metabolism. However, this provides limited information on the underlying cellular processes responsible for defining the response of the tissue to neurotoxins. It is widely accepted that many compensatory mechanisms that still lack a clear definition, are active within the NSDA pathway to maintain function, despite extensive neuronal loss that may derive from both experimental and clinical situations (Anglade *et al*., 1995; Finkelstein *et al*., 2000; Song & Haber, 2000; Zigmond & Hastings, 1998). To our knowledge, this is the first report of different 6-OHDA administration site-specific responses to NSDA toxins in the developing CNS. The current data add to emerging insights already gained regarding the compensatory mechanisms active in the developing CNS. However, the source of the DA pool that was revealed on the lesioned side and whether this source is only of a transient nature remains an interesting topic for future investigations.

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CHAPTER 5

MATERNAL SEPARATION EXAGGERATES THE TOXIC EFFECTS OF 6-HYDROXYDOPAMINE IN ADULT RATS: IMPLICATIONS FOR NEURODEGENERATIVE DISORDERS

1. INTRODUCTION

In the current study, we hypothesize that exposure to stressful stimuli during the early-life phases may predispose an individual to develop neurodegenerative diseases, specifically PD, during the later stages of life. During stressful situations various mechanisms, such as the hypothalamic-pituitary-adrenal (HPA) axis, are activated with the purpose of restoring the internal environment to its baseline activity (homeostasis). However, these mechanisms are designed for coping with the demands imposed by short-acting stressors only, and therefore chronic exposure to severe stress-provoking demands associate with the suppression of reproductive, growth, thyroid and immune functions, and this may lead to various pathological states.

It is acknowledged that the majority of individuals who experience stressful events, generally do not develop severe pathologies as a result of this. It is well documented that genetic factors may play a significant role in rendering certain individuals more vulnerable to stress and stress-related disease than others (Francis *et al*., 1999). This study intends to show that environmental factors such as early life stress may also be a significantly provoking factor in the development of neurodegenerative disorders

2. THE PHYSIOLOGICAL RESPONSE TO STRESS

An organism is incapable of surviving in the absence of the ability to adapt to its immediate environment. Homeostasis is constantly under threat due to exposure to a multitude of stressors. Therefore, the stress response is also referred to as “allostasis”, meaning to “maintain stability through change” (Sterling & Eyer, 1988). Virtually all physiological systems, including the cardiovascular, immune, respiratory, digestive, sensory, metabolic and nervous system are modified in order to meet the perceived danger during the body’s response to stress.

The initial response to stress involves an activation of the various bodily systems that play a part in the general alarm response raised to restore homeostasis. A critical component of this includes activating the HPA axis. Upon activation, energy is released from stored nutrients (i.e. triglycerides, glycogen) by transferring them into energy sources (free fatty acids, glycerol, glucose, amino acids) available for immediate use by the body. In addition, cardiovascular and

pulmonary tone is increased to facilitate delivery of oxygen and glucose to the tissue, while anabolic processes are slowed. Moreover, processes that are not vital for survival during the stress-crises, including digestion, growth, reproduction, inflammatory and immune responses are suppressed until the acute emergency has been survived. In prehistoric times, the physical changes in response to stressful stimuli were often an essential adaptation for avoiding the cost of natural threats, such as loss of life or sustaining severe injuries. In modern times, the stress response can be of benefit for raising performance levels during critical events, such as sports events, an important meeting, and/or when meeting situations of actual danger or crisis. In addition, cognitive processes are altered, tending towards lowered sensory thresholds and a logical adaptation for coping with an emergency situation.

Stress also triggers the release of certain neurotransmitters, such as adrenaline and noradrenaline. The released catecholamines activate the amygdala-region of the brain, which appears to trigger an emotional response, such as fear, to the stressful event. Neurotransmitters also signal the hippocampus, located in close proximity, to store the emotionally-rich experience in long-term memory, serving a survival-related role, to allow the organism to recognise the early signs relating to the particular danger during a future encounter, in order to avoid the danger and the adverse effects associated with this altogether.

The HPA constitutes the major neuroendocrine stress response system (Malarkey *et al*., 1995, Heim & Nemeroff, 2001), while the sympathetic-adrenal-medullary (SAM) system comprises the other important component in mediating the response. Both systems have a non-specific response pattern, although their activation threshold and time-course differs. (Pecoraro *et al*., 2006). Interestingly, while the short-term activation of the HPA axis allows adaptive responses to the challenge, in the long-run this can hold devastating consequences for the organism.

The neurons of the paraventricular nucleus (PVN) of the hypothalamus produce corticotropin-releasing factor (CRF). Projecting from the CRF cells to the median eminence the axons transport their contents to the primary portal vasculature where passive transport mechanisms diffuse these into the primary portal vasculature comprising of leaky capillaries. Vascular transport to the secondary plexus in the anterior pituitary occurs, whereupon CRF stimulates glandular corticotrope cells to synthesize pro-opiomelanocortin (POMC). This large molecule is processed by convertases in the cell to adrenocorticotropic hormone (ACTH) which is then stored in secretory vesicles. An additional immediate effect to CRF release is that stored ACTH is released directly into the systemic circulation system. The augmented plasma ACTH concentration then stimulates cells in the zona fasiculata of the adrenal cortex to secrete glucocorticoids into the peripheral blood circulation. Glucocorticoids act upon peripherally

located cells as well as CNS neurones that contain glucocorticoid receptors, which they reach after crossing the blood brain barrier (BBB) (Power & Schulkin, 2006).

3. EARLY LIFE AS A WINDOW OF DEVELOPMENTAL VULNERABILITY FOR DEVELOPING STRESS-INDUCED PATHOLOGY DURING LATER LIFE

Development represents a critical moment for shaping adult behavior and may set the stage to disease vulnerability later in life. The perinatal period shows enhanced plasticity, especially by the stress system, and is therefore particularly vulnerable to stressors. Exposure of the developing brain to severe and/or prolonged stress may result in hyper-activity of the stress system, defective glucocorticoid-negative feedback, altered cognition, novelty seeking, increased vulnerability to addictive behaviour and mood-related disorders (Charmandaru *et al*., 2003). Therefore, stress-related events that occur during the perinatal period can permanently change brain functions and the behaviour of the developing individual. For example, prenatal restraint stress (PRS) in rats is a well-documented model of early stress known to induce long-lasting neurobiological and behavioral alterations, including impaired feedback mechanisms of the HPA axis, disruption of circadian rhythms and altered neuroplasticity (Maccari & Morley-Fletcher, 2007). Specifically, PRS rats associated with hippocampal dysfunctions and several behavioral and endocrine disorders relating to this brain area. In view of estimates that between 5-16% of pregnant women could be exposed to physical or sexual abuse while they are pregnant (Cokkinides *et al*., 1999; Goodwin *et al*., 2000; Hedin & Janson, 2000; McFarlane *et al*., 1996), it is likely that a large proportion of children are exposed to prenatal stress.

A number of studies have shown strong associations between child maltreatment – either of a physical, emotional and/or a sexual nature – and various forms of mental disorder manifesting during childhood, adolescence and adulthood as major depression, posttraumatic stress disorder (PTSD), attention-deficit/hyperactivity disorder (for a review, see Famularo & Pelcovitz, 1992). In addition, a community-based study containing a database of over 2000 women reported that those with a history of childhood sexual or physical abuse, in the absence of a similar traumatic event occurring during adulthood, exhibited a higher frequency of depression and anxiety, with a higher rate of attempted suicides than women without such a history (McCauley *et al*., 1997).

Genetically sensitive ‘natural experiments’ had been important in showing that environmental mediation was a highly likely factor in the behavioral outcome seen. Discordant twin designs showed that the mental disorders in adult life were much more likely to arise in the twin who suffered sexual abuse than the twin who did not. Moreover, the strength of association was very comparable to that found in the population as a whole (Kendler & Prescott, 2006). Multivariate twin analysis (Jaffee *et al*., 2004) also proved crucial for showing a marked difference between corporal punishment (with no evidence of environmental mediation detected) and maltreatment.

However, these studies usually implicate adverse exposure during *adulthood*, while only limited reports propose a neurodevelopmental basis for neurodegeneration. Environmental exposures during early life pose an enhanced risk due to the CNS receiving exposure to neurological insults during critical periods of vulnerability. These include the possible effects that certain teratogens may have on the nigrostriatal dopaminergic system, including exposure to agrichemicals (Barlow *et al*., 2004), an iron-enriched diet (Jones *et al*., 2002) during the perinatal period, or exposure to maneb (Cory-Slechta *et al*., 2005), cocaine (Glatt *et al*., 2004) or bacterial endotoxin lipopolysaccharide (LPS) (Ling *et al*., 2006) during the prenatal period. In addition, a highly concentrated prenatal endocrine environment, characterized by high levels of glucocorticoids, seems to have detrimental effects on the long-term survival rate of cerebellar granule cells, leaving these cells more susceptible to the effects of oxidative stress (Ahlbom *et al*., 2000).

3.1. Physical and sexual abuse of children as a source of stress

Children subjected to physical or sexual abuse are at greater risk to develop psychiatric problems later in life (Bifulco *et al*., 1991). The definition of abuse is multifold and includes persistent emotional neglect, without necessarily including physical abuse as well, to act as a risk-factor for developing depression during adult-life (Canetti *et al*., 1997). Such vulnerability might reflect rearing conditions exposed to during early postnatal life (Brake *et al*., 2004). During normal rat mother pup-interactions, the dam leaves the litter routinely for only 20-25 min. at a time. Thus, by extending this period of separating the dam from her pups, a model deprivation of maternal care is created and applied to the human species. However, separating the mother from the pups should not be regarded as an extreme form of deprivation, since it is not unusual behaviour to observe the dam leaving the litter for up to 3 hrs to forage for food when in the wild (Calhoun, 1962). However, the difference between separation as a natural phenomenon and when experimentally induced, is that, in the case of maternal separation as an animal model, dams are forced away from their litters and are unable to hear vocalizations from the pups.

Experiments performed on rats show that disrupted normal care during this period can have chronic effects on the normal neuroendocrine response to environmental stress. More specifically, repeated exposure of animals to periods of MS during the first 2 weeks post-birth resulted in an increased stress reactivity that can last across the animal's entire lifespan (Plotsky & Meaney, 1993; Liu *et al*., 2000). During adulthood, rats subjected to maternal separation during the postnatal period display increased fearfulness, greater HPA axis and behavioral responses to stressors, as well as increased locomotor activity in a novel setting (Piazza & Le Moal, 1997).

3.2. Early-life exposures to stressful events as a trigger for adult neuropathogenesis

Stress can be defined both as an internal or external stimulus (as described above), or as a non-specific adaptive response by the body to any demand, leading to rapid changes affecting the entire body (Harshpinder & Aujila, 2006). These adaptations serve to restore a state of homeostasis when this is threatened, or perceived to be threatened (Selye, 1949) that can prove life-saving in a critical life-or-death situation. However, with extended periods of time, exposure to repeated stressful situations place excessive strain on the body that may contribute substantially towards developing medical problems of both a physical and psychological kind. Clinical conditions associated with exposure to severe or chronic forms of stress (e.g. post-traumatic stress disorder and depression) have been found to demonstrate signs of neuronal degeneration, including evidence of atrophy in the hippocampus (Sapolsky *et al*., 1996; Bremner *et al*., 1997). Similarly, the subjection of rodents and non-human primates to chronic levels of restraint stress (Watanabe *et al*., 1992), chronic multiple stressors (Magarinos & McEwen, 1995), or psychosocial stress (Uno *et al*., 1989; McKittrick *et al*., 2000; Czeh *et al*., 2001), resulted in degenerative changes within hippocampus CA3 pyramidal neurons.

The aetiology of neurodegenerative disorders, including PD, continues to receive intense investigation, with the aim of developing improved treatments. However, although the precise mechanism by which PD is induced remains unknown, both genetic susceptibility and exposure to adverse environmental conditions are regarded as major risk factors for developing the disease (for a recent review, see Yamashita & Matsumoto, 2007). The idea that PD is environmentally influenced seems feasible due to evidence that an inflammatory reaction by the CNS's resident immune cells, the microglia and astrocytes, when exposed to noxious elements during early life may be a possible cause or contribute to developing PD in later life (Liu *et al*., 2003).

Although many individuals experiencing stressful events do not develop pathologies, chronic stress seems to be a triggering factor in those individuals that are particularly vulnerable. There is evidence to suggest that the neuroendocrine response to stressful stimuli may act as a causative factor to neurodegenerative diseases, with evidence for a possible genetic link between the intensity of the stress response, the rate of age-dependent neurodegeneration and the individual's life expectancy (Glad & Gilad, 1994). For instance epigenetic factors may permanently increase HPA axis activation (Tsankova *et al*., 2007).

4. RESEARCH HYPOTHESIS

To expand current knowledge of environmental impact on the state of DA neurons, the present study investigated whether exposure to stress early in life could predispose individuals to the development of a neurodegenerative process such as PD. To test this hypothesis, rats were maternally deprived for 3 hrs a day between postnatal days (PND) 2-14, and injected with a single unilateral injection of 6-OHDA into the striatum when at 35 days of age. We specifically opted to lesion the rats when they reached an adolescent stage (at 35 days of age), since the synthesis-rates of striatal D_1 and D_2 DA receptors reach their apices at this stage (Andersen, 2002). The motor performance of the animals was assessed at 28 days following surgery using a series of behavioral measures, and the results compared to a group of rats that had not previously been exposed to MS prior to toxin exposure. Following the behavioral assessment, the rats were decapitated and the brain tissue collected for immunohistochemical analysis.

5. MATERIALS AND METHODS

5.1. Animals

Sprague-Dawley rats were used to generate the pups for this study. These animals were obtained from the Central Animal Research Facility of the University of Cape Town. The animals were housed in 40 × 25 × 20 cm acrylic cages (4 rats per cage) with pine wood shavings that covered the cage floor. Rat chow and tap water were available *ad libitum*. Temperature was maintained at 22˚C and lights were on each day from 06:00 to 18:00. The experimental procedures were approved by the Committee for Experimental Animal Research of the University of Cape Town (project number: 006/008) and were also in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

5.2. Maternal separation paradigm

Maternal Separation (MS) has been described as a potent naturalistic stressor (Stanton *et al*., 1988) and has been used extensively as a model of early life stress in our laboratory (Daniels *et al*., 2004; Faure *et al*., 2006), as well as by many others (Ladd *et al*., 1996; Lubach *et al*., 1995; van Oers *et al*., 1998; Lyons *et al*., 1999; Sánchez *et al*., 2001). Adult male-female pairs were housed under normal conditions to allow for mating. Immediately after the rat pups were born, the adult males were removed, while the mothers remained with the pups. Litters were sexed and culled to 8 pups per dam. If there were fewer than 8 males, then females were included to complete the group to ensure equal nurturing. The various dams with their pups were randomly assigned to either a control (non-separated) or to an MS group. MS involved physically removing the dam from the home cage that contained the pups for 3 hrs per day from 09:00 to 12:00 beginning on PND 2, and leaving the pups undisturbed in their home cage. During the separation period, the pups were housed in a room away from the mother to prevent

communication *via* ultrasound vocalizations from taking place (Hofer, 1994). The temperature of the room to which the rat pups were transferred was maintained between 31˚C and 33˚C, thereby minimizing the possible influence of hypothermia. After PND 14, the pups returned to normal housing conditions. Control animals were handled identically to experimental ones, except that the pups were not separated from the dam during the first 14 days after they were born. On PND 21 both groups were weaned by permanently removing them from the dam.

5.3. Preoperative and surgical procedures

Surgery occurred at PND 35. To prepare the surgical site, the scalp hair was clipped and the site disinfected with 70% ethanol. Body heat was conserved during surgery by placing the rat on a heating pad (Rex C10, Centre for Electronic Services, University of Stellenbosch). The rats (n = 16) were anaesthetized using a combination of ketamine hydrochloride (Anaket-V®, Bayer Healthcare, South Africa) and medetomidine hydrochloride (Domitor®, Pfizer, South Africa) (0.1 ml/100g, i.p.) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, USA). Bregma and Lambda were used to level the horizontal plane of the skull. From bregma, the following coordinates were used: anterior, 0.30 mm; lateral, 2.7 mm; ventral, 5.6 mm below the dura mater. A small burr hole was drilled at the determined coordinates and a 22-gauge surgical-steel cannula was slowly inserted into the brain. A 6-OHDA solution was freshly prepared, kept on ice, and protected from exposure to light. The solution consisted of 12 µg (free-base) 6-OHDA (Sigma, St. Louis, USA), dissolved in 4 µl 0.1% L-ascorbic acid in 0.9% sterile saline, and was administered unilaterally into the left striatum, using a 341-A model syringe pump (Sage Instruments, USA), running at an injection rate of 0.5 µl/min. The cannula was left in place for an additional 5 min following the infusion to facilitate the optimal diffusion of the solution. Subsequently, the needle was slowly retracted from the brain. The hole drilled in the skull was filled with sterilized oxidized cellulose (Ethicon, UK), the incision sutured, and lastly coated with an iodine solution (Purdue Frederick, USA). The rats were placed in individual cages under heating-lamps until full recovery was achieved, after which they were returned to their home cages.

The unihemispheric administration of the neurotoxin 6-OHDA to rodents is used frequently for studying various aspects relating to PD, including disease aetiology (Schwarting & Huston, 1996; Ungerstedt, 1971), the potential therapeutic benefits of brain grafts (Curran *et al*., 1993; Dunnett *et al*., 1983), as well as the implications of other agents, including neurotrophic factors (Winkler *et al*., 1996; Zigmond & Keefe, 1997). The toxic effects of 6-OHDA are considered species-dependent, with severe parkinsonism induced by administering the toxin to rats, but seemingly resolving spontaneously after 4-6 weeks when infused in rats (Rothblat *et al*., 2001). In addition, the effects of the lesion may also be strain dependent, since

Lewis rats require a two-fold higher dose of 6-OHDA to evoke the same behavioral change as seen in Fischer and Sprague-Dawley rats (Collier & Sortwell, 1999; Flores *et al*., 1998).

Furthermore, using a particular mouse strain, Hefner *et al.* (2007) were unable to produce robust behavioral changes following exposure to periods of MS, to indicate that the effects pertaining to MS might also be species-constrained. Hence, my decision to use Sprague-Dawley rats in this particular study was influenced by the above findings.

5.4. General behavioral testing procedures

A number of behavioral tests were done to ascertain the degree of asymmetry following the 6-OHDA injection. These tests were performed between 09:00 and 13:00, 28 days after the intra-striatal injection.

5.4.1. Vibrissae-evoked forelimb placing test

Rats were subjected to the vibrissae-evoked forelimb placing test as previously described by Schallert *et al.* (2000). See chapter 3 (5.2) for a complete description of the methodology followed in performing this test. Independent testing of each forelimb was induced by carefully brushing its vibrissae against the edge of a tabletop. Each vibrissae brush constituted a trial, with 10 trials from each side being performed. If the rat placed its forelimb swiftly upon the table's surface immediately following vibrissae stimulation, a score of 1 was allocated, while a failure to place the paw on the table provided a score of zero.

5.4.2. Single-limb akinesia (Bracing) test

This test evaluates the animal's capacity to adjust its stepping to regain postural stability, when weight-shifts are imposed by the experimenter (Schallert *et al*., 1979; Lindner *et al*., 1995; Schallert *et al*., 1992; Olsson *et al*., 1995). The animal was held by its torso with its hindquarters and one forelimb lifted above the surface of a table to allow the weight of its body to be supported by one forelimb alone. The animal was then slowly moved in the forward direction. The number of adjusting steps was recorded as the animal was moved in 30 sec trial periods. The test was performed once for each forelimb.

5.4.3. Small open field test

The rat was placed in an upright transparent acrylic cylinder (20 cm in diameter and 25 cm in height). The cylinder height was such that the animal was unable to reach the top edge when rearing and wide enough to permit a 2 cm space between the tip of its snout and the base of its tail when all four paws were placed on the floor. Care was taken for the rats not to habituate to the cylinder lest they should become inactive. This was done by restricting the period of testing

to 5 min (Schallert *et al*., 2000) and by placing the rats back in their home cage between the behavioral tests.

A defecation and urination index was also determined based on the number of defecation boli and urination spots observed. The number of rearing (standing on hind limbs without touching the wall of the cylinder) was also recorded. These parameters served as robust measures of the emotional status of the animals (Gray & Cooney, 1982; Pijlman *et al*., 2002).

5.5. Animal euthanasia and tissue collection

Following the behavioral assessment on PND 63, the rats were decapitated and the left and right striatum rapidly dissected on ice. The animals were anaesthetized with Halothane™ (Safeline Pharmaceuticals, South Africa), after which 60 ml of phosphate buffered saline (PBS) was used to exsanguinate the animals via transcardial perfusion. This was followed by 180 ml of 4% paraformaldehyde-PBS (Merck, South Africa) to fix the brain tissue. The brains were rapidly removed from the skull and the tissue placed in 30% sucrose solution. Following this, the brain tissue was blocked and frozen over liquid nitrogen vapor until sectioning.

5.6. Tyrosine hydroxylase (TH) immunohistochemistry

The effect of the injections on tyrosine hydroxylase immunoreactivity (TH-ir) was evaluated in 3 rats belonging to each group. To stain for TH, free-floating coronal sections 60 µm thick were cut with a cryostat at -20 °C. The sections were quenched by incubating with 0.3% hydrogen peroxide for 30 min at room temperature in a humidifying container. This was followed by a succession of washes (5 ×10 min) with PBS (0.15 M, pH 7.4). The sections were placed in a 10 mM citrate buffer (pH 6.0) and boiled in a microwave oven for 15 min. The sections were left to cool, before the wash-cycle was repeated. The sections were then pretreated with 150 µl horse blocking serum and 0.1% Triton X-100 for 30 min before application of the primary monoclonal anti-TH antibody (dilution 1:500, DiaSorin, USA) and overnight incubation at 4ºC. The next day the slices were washed in PBS (5 ×10 min), before applying the secondary biotinylated antibody (dilution 1:400) for 1 hour at room temperature. The wash routine was repeated before incubating the slices with peroxidase labeled Mouse IgG ABC reagent (ABC kit, DakoCytomation, USA) with concentrated diaminobenzidine (DAB) as the chromogen. Tissue sections were incubated at room temperature until staining developed. The sections were given a final 15 min rinse with distilled water, before dehydrating them with 96-100% ethanol, clearing with Xylol (Merck, South Africa) and mounting the sections onto clear glass slides.

5.7. Quantification of relative TH immunoreactivity

We adopted the method of Anstrom *et al.* (2007) to assess the extent of the damage caused by

the unilateral 6-OHDA injection. Sections representing the anterior, middle, and posterior striatum from 3 control and 3 maternally separated animals were analyzed. Digital photographic images of all the slices were captured under constant, standardized lighting conditions and exposure using the 10x objective on a Zeiss microscope fitted with Brightfield optics and a Diagnostic Instruments Spot Insight QE camera. For each hemisphere, 3 independent digitalized photographic images were produced (Fig. 7). This was done in a consistent manner anchored by objective landmarks (anterior commissure dorsally, optic chiasm ventrally, and the third ventricle medially). The degree of neurochemical asymmetry of each image was determined with Zeiss's Axiovision image analysis software. The TH-ir surface area of the image in the 6-OHDA-infused hemisphere was divided by that of its corresponding image in the non-infused hemisphere. The ratio of TH-ir on the lesioned side to TH-ir on the non-lesioned side was expressed as a percentage. Values closer to 100 indicate minimal loss of TH staining, while values closer to zero indicate severe neurochemical asymmetry. The percentage values of the 3 images were averaged to give an indication of damage per section, and averaging the percentages of the 3 sections for a given rat provided the degree of neurochemical asymmetry for a specific animal. The final value was calculated by averaging the scores of the 3 individual rats per group. See figure 1 for an example of such an analysis.

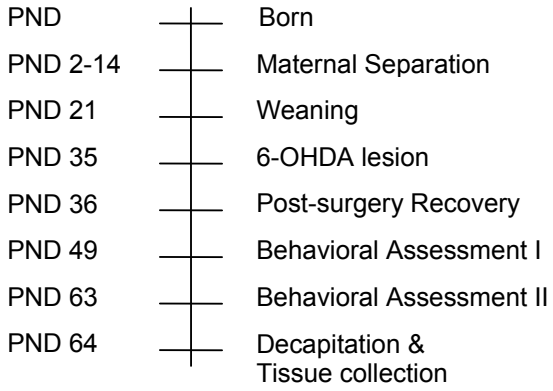

Fig. 1. Sequence of maternal separation, lesioning and behavioral testing. The time-points are given in days.

5.8. Statistical analysis

The statistical analysis of all the data was performed using Graphpad Prism V.4 (USA). For analyzing the behavioral data, comparisons between groups were performed using the Kruskal-Wallis test, followed by Dunn's Multiple Comparison's test, while the Mann-Whitney test was

applied when only two groups were compared. To analyse the results of the TH-stain analysis, a student's *t*-test was used. Data is expressed as the mean ± S.E.M. and results are considered to differ significantly if $p < 0.05$. For the statistical analysis of the HPLC results, data were subjected to a parametric unpaired student's *t*-test. All the results of analysis were considered to differ significantly if $p < 0.05$, and data were expressed as the mean ± S.E.M.

6. RESULTS

6.1. Behavioral results

6.1.1. Vibrissae-evoked forelimb placing test

A number of behavioral tests was employed at 28 days after lesioning to evaluate the effect of 6-OHDA on the locomotor abilities of rats. This was achieved by comparing ipsilateral limb movements to those of the contralateral limb. For the vibrissae-elicited forelimb placing test, a failed response was defined as the inability of the rat to place its paw on the tabletop following stimulation of the vibrissae. 6-OHDA produced a 65% increase in the number of errors made by the contralateral paw as compared to the ipsilateral paw ($p < 0.01$). Moreover, the number of failed responses with the contralateral paw was 100% greater in the MS group than in the non-MS group ($p < 0.05$; Fig. 2).

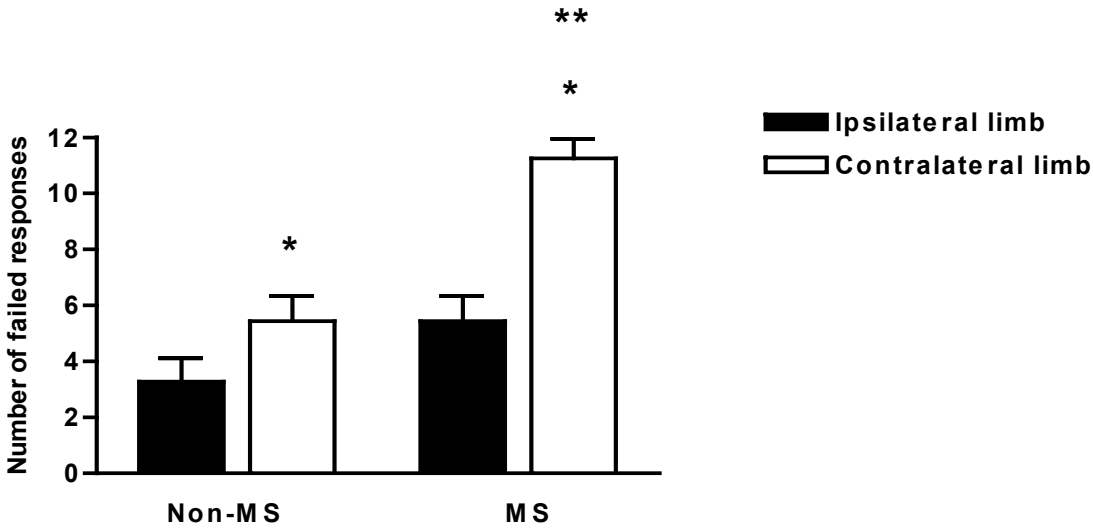

Fig. 2.

Failed responses in non-MS and MS rats during the vibrissae-evoked forelimb placing test, performed at 28 days after receiving a unilateral 6-OHDA injection into the left striatum. *$p < 0.01$

indicates a significant difference from the number of failed responses made by the ipsilateral limb, while **$p < 0.05$ indicates a significant difference between the number of failed responses of the contralateral limb of the non-MS and MS groups (Kruskal-Wallis test, followed by Dunn's Multiple Comparison test). Data represent the mean ± S.E.M ($n = 15$).

6.1.2. Single-limb akinesia (Bracing) test

The bracing test showed that in non-MS animals the number of steps made within a 30 sec time interval along a straight line by the contralateral limb was significantly decreased by 6-OHDA, when compared to its ipsilateral limb ($p < 0.01$). The MS rats revealed a significantly greater deficit in use made by the contralateral limb when the results were compared to the non-MS rats (62% versus 39%, respectively, $p < 0.05$; Fig. 3).

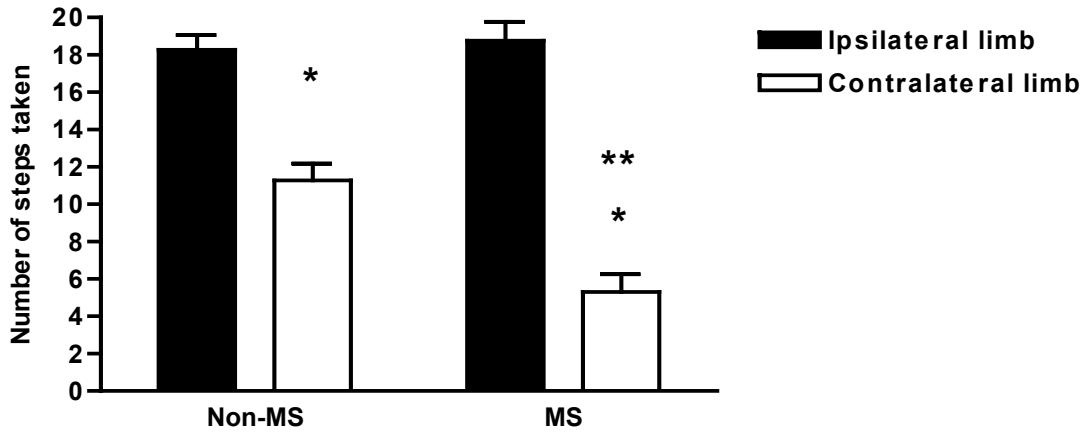

Fig. 3. The number of steps taken by the contralateral limb compared to the respective ipsilateral limb during the bracing test, performed at 28 days after a unilateral 6-OHDA injection into the left striatum. Fewer steps where taken by the limb contralateral to the 6-OHDA-induced lesion, and this reduction was exaggerated in the MS group compared to the non-MS group (*$p < 0.01$; **$p < 0.05$; Kruskal-Wallis, followed by Dunn's Multiple Comparison's test). Data represent the mean ± S.E.M ($n = 15$).

6.1.3. Small open field test

6.1.3.1. Defecation-urination index

The small open field test revealed that rats subjected to MS had a significantly ($p < 0.0001$) greater defecation-urination index (2.88 ± 0.63) than non-MS animals (1.27 ± 0.43) (Fig. 4).

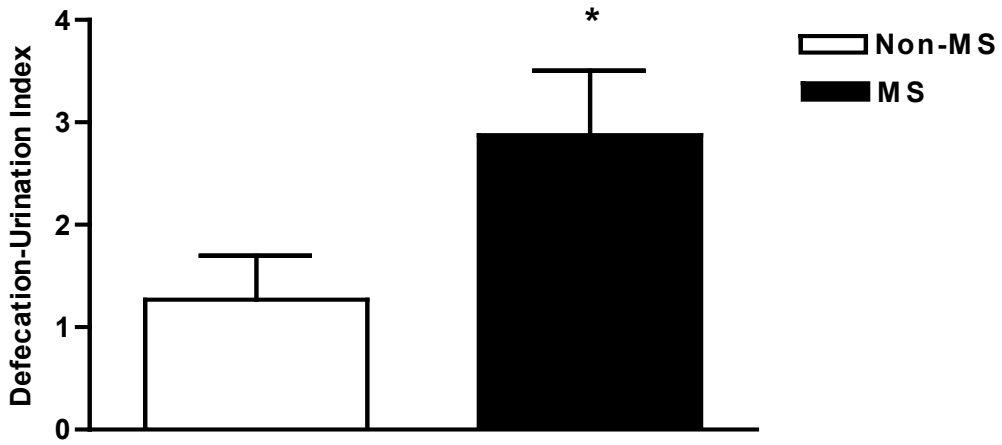

Fig. 4. The defecation-urination (DU) index obtained in the small open field test. As shown above, the index of the MS animals are significantly higher compared to the non-MS animals (*p < 0.0001, Mann-Whitney test). The data represent the mean ± S.E.M (n = 15).

6.1.3.2. Frequency of rearing

In addition, MS animals display a significantly (p < 0.0001) higher frequency of rearing (2.68 ± 0.27) than the non-MS controls (1.0 ± 0.21) (Fig. 5).

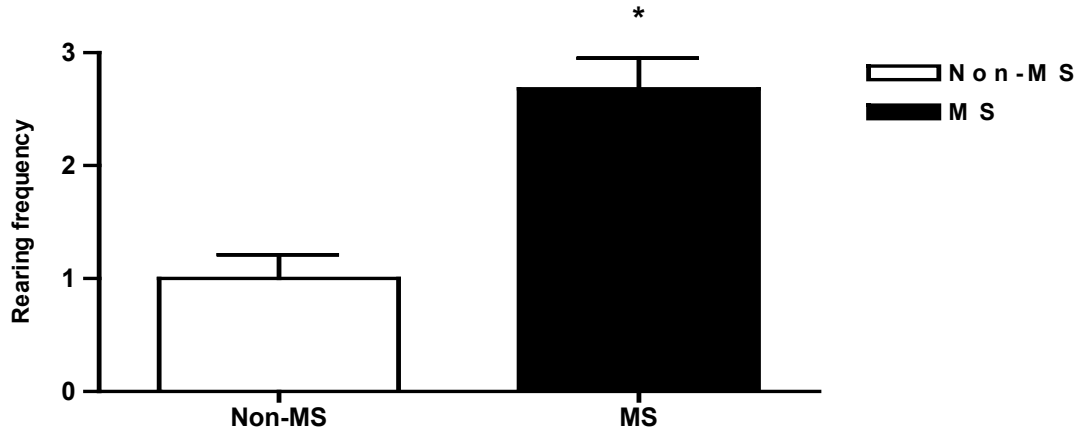

Fig. 5. The increase in rearing frequency displayed by MS rats compared to non-MS ones during the cylinder test. Data represent the mean ± S.E.M (n = 15). *p < 0.0001, Mann-Whitney-U test.

6.2. Analysis of Tyrosine hydroxylase (TH) density staining

The extent of the intrastriatal lesions were qualitatively assessed by investigating the loss of TH-ir fibers in coronal sections of the forebrain using light microscopy. The 6-OHDA injections produced a consistent and distinct lesion, as reflected by a substantial decrease in immuno-

staining in the lesioned striatum compared to the contralateral side. There is a significantly greater loss of TH-ir in the striatum of the MS rats compared to controls that reached statistical significance ($p < 0.05$; Fig. 6).

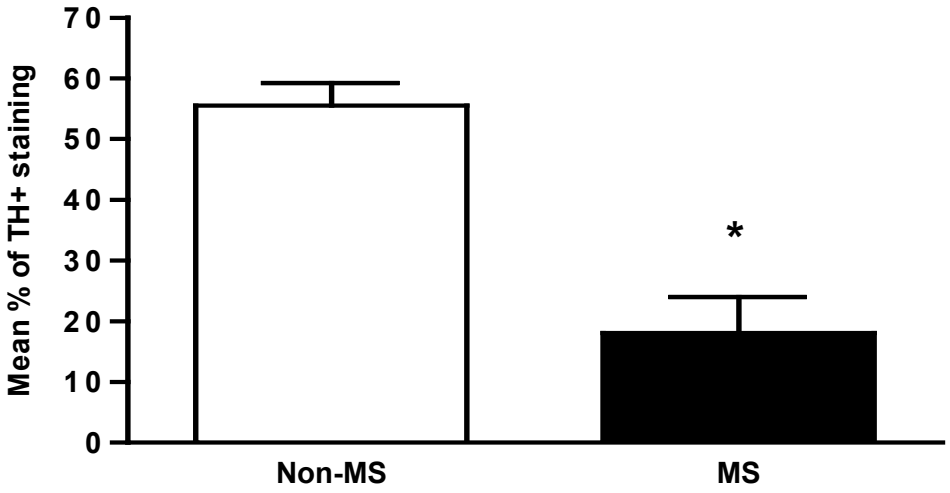

Fig. 6. TH staining detected in striatal sections of MS and non-MS rats. TH staining percentages were calculated by expressing total degree of TH staining of the lesioned hemisphere in relation to the stained area of the non-lesioned control side. The percentage TH staining was significantly less in MS animals compared to the non-MS group (*$p < 0.05$; Mann-Whitney-U test). Data represent the mean ± S.E.M. ($n = 3$).

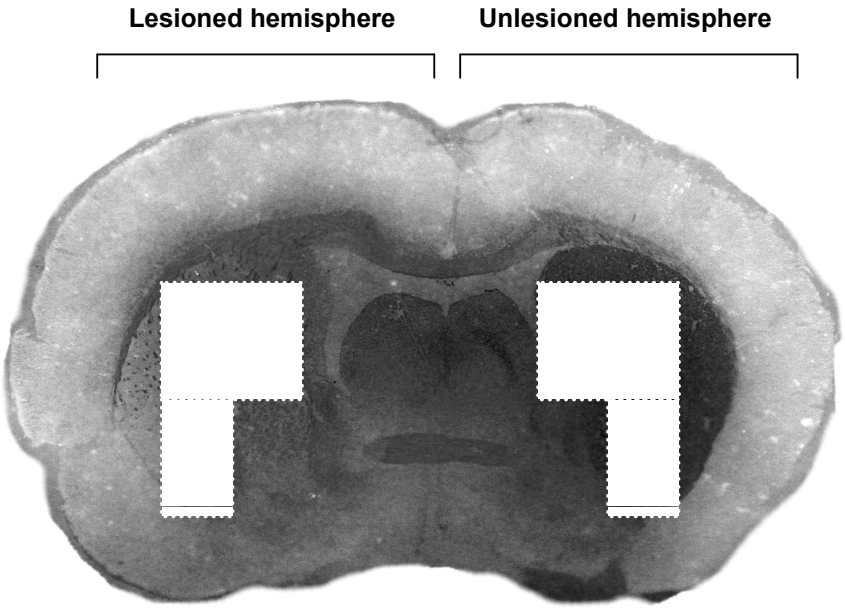

Fig. 7. A photomicrograph showing decreased TH staining in the lesioned (left) striatum following a unilateral 6-OHDA injection. The broken lines depict the 3 sample areas per hemisphere that were used to quantify TH stain density using Zeiss K3100 (v.4) analyzing software.

7. GENERAL DISCUSSION

The experimental strategy for the current study was to subject rat pups to MS as the stressful early life event, and then to investigate its effects on 6-OHDA-induced alterations in locomotor activity and DA neuron loss. The results shows that animals subjected to MS and having undergone 6-OHDA lesions experience exaggerated locomotor deficits compared to normally reared controls. Furthermore, stressed animals displayed poorer performance in sensorimotor tests, such as the vibrissae and bracing tests, a higher defecation-urination index and rearing frequency, and an apparently greater 6-OHDA-induced loss of DA terminals in the striatum as reflected in augmented loss of TH-ir. These findings may relate to clinical reports that stressful experiences can worsen the degree of motor-related impairment in PD patients, such as increased tremor when patients express anger or anxiety (Schwab & Zieper, 1965), and that experiencing severe bouts of stress may even exacerbate the underlying neuropathology (Smith *et al*., 2002).

Mesencephalic dopaminergic neurons function as the most important source of DA in the mammalian CNS, and their loss lead to both the motor-associated symptoms commonly associated with PD. Although they are few in number, they play a critical role in regulating various brain functions, including voluntary movement and a broad array of other processes, such as mood, reward, addiction and stress (Lim, 2005). The motor symptoms that were observed during this study may be the result of the pulsatile stimulation of the dopamine receptors, to provide just enough in order to observe a behavioral deficit, or it may be possible that either no recovery occurred or that degeneration continued at a rate sufficient to mask ongoing plasticity. Consistent with the findings of others (Glavan & Zivin, 2005), a near total loss of TH-positive fibres was observed on the hemisphere side that had been treated with 6-OHDA, due to the substantial decrease in TH-positive neuron numbers. This provides a platform on which to argue that particular subpopulations of the affected neurons are better adapted to survive under such toxic insults, by providing a buffering capacity towards fluctuating dopamine levels. Our results therefore imply that synaptic plasticity, in response to the administration of 6-OHDA, is exacerbated and involve a complex pattern of changes in the surviving dopaminergic population, to result in an increase in firing potential and the heightened release of DA to near-normal levels. This phenomenon may result from increased expression of the neurotrophic factors, especially of the neurotrophins that provide for increased expression of trophic agents in such young animals. In particular, BDNF has been implicated to play a fundamental role in regulating neuronal survival, differentiation and synaptic plasticity (Baquet

et al., 2005). Further relevance for this statement is provided in that reduced levels of BDNF expression has been detected within the substantia nigra in patients suffering from PD.

In studies of 6-OHDA-treated rats, compensatory adaptations in the remaining DA neurons have been observed, with rats often completely recovering from the gross motor deficits ordinarily expected from a larger dose or even not manifesting any gross motor symptoms at all (Abercrombie *et al*., 1990; Robinson *et al*., 1994a; b; Fornaquera & Schwarting, 1999). However, studies have also shown that the presentation of added stressors to these seemingly recovered animals often results in sustained neurological impairments (Stricker & Zigmond, 1974; Snyder *et al*., 1985). These findings are consistent with clinical reports that stressful experiences can worsen the degree of motor-related impairment in PD patients, such as increased tremor when patients express anger or anxiety (Schwab & Zieper, 1965), and that experiencing severe bouts of stress may even exacerbate the underlying neuropathology (Smith *et al*., 2002). Sapolsky (1992) coined the term “neuro-endangerment”, to describe the phenomenon that exposure to stressful events can increase the vulnerability of neurons to a subsequent insult.

The current finding that MS rats display greater impairments than controls after 6-OHDA lesioning could reflect a direct effect that early life stress has on neuronal function. Zhang *et al*. (2002) found, for example, that maternal deprivation enhances cell death in the cerebral cortex and hippocampus.

Thus, MS may have increased the vulnerability of DA neurons to the oxidative stress associated with 6-OHDA. Consistent with this is our finding that staining for striatal TH was significantly lower in the lesioned hemisphere of MS rats than in control animals, suggesting greater DA cell loss in the 6-OHDA-treated animals. Rearing conditions have been shown to induce long-lasting alterations in the central monoaminergic systems of the brain (Daniels *et al*., 2004), particularly DA levels (Arborelius & Ecklund, 2007; Miura *et al*., 2002), DA receptor density (Ploj *et al*., 2003), and decreased DA turnover (Matthews *et al*., 2001). Although these changes have been reported for brain areas other than the SN or the striatum, it raises the possibility that similar alterations could also occur within the nigrostriatal system.

Since it has been shown that the cell-mediated immune status of animals are affected by exposure to early social deprivation, with evidence of a decreased ratio of helper to suppressor T cells (Lewis *et al*., 2000), compromised immune function may also have played a role in the augmented neurological impairments seen in the MS rats. One mechanism may involve biochemical pathways affected by glucocorticoids, since it is well accepted that stress can trigger increased glucocorticoid release into the peripheral circulation. Daniels *et al*. (2004) and others (Plotsky & Meaney, 1993; Ladd *et al*., 2000; Slotten *et al*., 2006) have previously shown that early life stress results in a deregulated HPA axis, reflected by a blunted response to

subsequent stress and high circulating corticosterone levels. Although this biochemical alteration may initially serve to improve focus, information processing, and the response to the stressful event, an excessive amplitude and duration of such changes may lead to neuronal cell death (Sapolsky, 2000). In accordance with this possibility, administration of corticosterone to adrenalectomised rats has been shown to increase the size of neuronal lesions induced by the injection of quinolinic acid into the striatum (Ngai & Herbert, 2005), and to decrease GC receptor density (Morale *et al*., 2006). Moreover, the key role played by GCs in stress-induced neuropathology has been shown, particularly for the hippocampus, to reduce the ability of some cells to survive toxic threats. Mechanisms by which this may occur include reduced energy metabolism, initiated through an inhibition of glucose uptake (Horner *et al*., 1990), by increasing extracellular Ca^{2+} signaling (Han *et al*., 2005), decreased expression of neurotrophic factors, such as brain-derived neurotrophic factor (Cosi *et al*., 1993; Schaaf *et al*., 1998; Lippmann *et al*., 2007), or by overstimulating apoptosis, through reduced expression of anti-apoptotic proteins and increased expression of pro-apoptotic proteins (Almeida *et al*., 2000; Cárdenas *et al*., 2002; Sapolsky, 2000).

A significant difference between maternally separated and normally reared rats was detected in terms of the defecation-urination index, with the maternally deprived rats obtaining a higher score. Spontaneous micturition following bladder distension does not occur within the first 10 PNDs of a rat pup's life. During this period, urination is usually dependent on perigenital stimulation by the mother (Maggi *et al*., 1986). It is therefore reasonable to predict that subjection of rat pups to MS may result in a disruption in the development of the micturition reflex (Wu & de Groat, 2006). Our data are contrary to this expectation, perhaps because following the 3-hour separation period the mothers immediately attended to the pups upon reunion (personal observation). This additional attention could have enhanced the development of the micturition reflex in our animals. However, the enhanced defecation of the MS animals correlates with the findings of others who also report enhanced colonic motor function (Coutinho *et al*., 1999; Daniels *et al*., 2004). The increased defecation suggests that the MS animals were more anxious than their normally reared counterparts.

The physiological basis for this effect may well relate to the increased vulnerability of the MS animals to 6-OHDA-induced degeneration and behavioral dysfunction. When a relatively smaller amount of 6-OHDA is administered, compensatory adaptations in the remaining DA neurons are observed, with rats often completely recovering from the motor deficits ordinarily expected from a larger dose or even not manifesting any gross motor symptoms at all (Robinson *et al*., 1994a; Fornaquera & Schwarting, 1999). However, studies have shown that when challenging stressors are presented to these seemingly recovered animals, they often manifest with neurological impairments (Stricker & Zigmond, 1974; Snyder *et al*., 1985).

Hind-limb rearing is important for the survival of the animal as it allows the rat to orient itself (Colorado *et al*., 2006) and examine its environment, to seek new sources of food, shelter or mating partners (Mällo *et al*., 2007). The significant increase in rearing in MS rats could therefore be interpreted as positive exploratory behaviour. Alternatively, the animal may experience rearing as a response to perceived personal threat, especially when escape routes are sought from a perceived hostile surrounding. In addition, exposure to predatory onslaughts may increase during this activity, thereby rendering the animal more fearful. Under these circumstances elevations in rearing frequency may reflect a state of hyperarousal and hence anxious behaviour (Uys *et al*., 2006). It is acknowledged that other measures, such as the standard open field and the elevated-plus maze are more commonly used to determine the degree of anxious-like behaviour in rats, than the small open field. Yet, whilst these two parameters used in the small open field may not be sufficient to clearly define the emotional status of MS animals, these suggest behavioral differences between the two groups. As in the case of the increased defecation-urination index, the basis for the increased rearing may relate to the mechanism underlying increased 6-OHDA toxicity.

8. CONCLUDING REMARKS

In summary, exposure to early life stress exacerbated the toxic effects of 6-OHDA on locomotor activity and decreased the degree of TH expression determined on the lesioned hemispheric side. Whether the effects observed are directly due to the effects that separation stress has on the central dopaminergic system, or whether it is induced indirectly via other centrally acting factors are uncertain. Further investigation will be required to elucidate the mechanism of the effects observed. Such investigations are well advised, as they will provide important insights into the means by which early adversity can increase the risk of developing a neurodegenerative condition such as PD. Furthermore, since it is widely reported that the immediate effects that exposure to unavoidable physical stress or even merely participating as a witness to these traumatic events has on animal physiology includes elevated corticosterone plasma levels (Weinstock, 2008), hyperthermia (Kikusui *et al*., 2001), and the suppressed activity of killer T- and B-cells (Kimura *et al*., 2005) future work should assess the degree of change in these analytical measures. Lastly, the possible influence of additional factors such as the concentration of neurotrophins and/or adrenocorticotropin (ACTH) and corticosterone were demonstrated in chronically stressed rats compared to normal-reared animals through a decreased expression of BDNF observed in the hippocampus and striatum, as well as augmented plasma ACTH and corticosterone concentrations (Lippmann *et al*., 2007). Although the current work did not include measures of neuro-endocrine function, it highlights the impact that adverse experiences during development may have in rendering neurons more susceptible

to subsequent insults. The possible contributions that decreased levels of neurotropins such as BDNF and GDNF, as well as increased may have made to the currently reported findings was further investigated in follow-up work, since molecular adaptations may induce alterations in neural circuitry that may affect the risk of developing a neurodegenerative condition, such as PD. In addition, we conclude that the toxin 6-OHDA acts as a robust inducer of neurogenesis and in striatal cellular plasticity in the rat lesioned during a young age, to protect the surviving neural microenvironment from damage, thus acting as an effective compensatory mechanism. However, in the case of those that have experimentally been subjected to MS stress, this physiological and behavioral mechanism has proved to be less effective.

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CHAPTER 6

NEUROPROTEOMICS AS A NOVEL TOOL IN PARKINSON'S DISEASE RESEARCH

1. INTRODUCTION

This chapter discusses the potential offered by the technological advances made in proteomics in providing a clearer understanding of the pathogenesis as well as revealing possible new targets for therapeutic intervention. It has already given insight into the role played by the aggregation and deposition of proteins in human PD and in chemically induced models of the disease (Schulenborg, 2006). In addition, it can reveal the patterns of PD-specific cellular markers to provide for earlier diagnosis and more effective tracking of treatment responses. Therefore proteomics is seen to allow the therapeutic regime to start earlier, when it is likely to have a more beneficial effect.

At present, the tools to diagnose PD preclinically are not yet available to clinicians (Montgomery *et al*., 2000a; Montgomery *et al*., 2000b). It is foreseen by many that the emerging field of neuroproteomics will allow for detecting new biomarkers with which to diagnose PD more accurately and earlier, thereby offering the possibility for early intervention (Zetterberg *et al*., 2008). This could allow the technological barriers imposed on previous studies to be overcome. By studying the unique expression pattern and level of PD-associated proteins, it is hoped that PD can be differentially diagnosed and insight gained into the mechanisms underlying this disorder. In addition, it may provide much-needed surrogate end-points with which to validate the clinical efficacy of novel treatment options (Michell et al., 2004).

2. A BRIEF OVERVIEW OF PROTEOMIC TECHNIQUES

The technology that is broadly described under the umbrella-term 'proteomics', derives from the term "proteome", that refers to "the proteins expressed by a particular genome" (Wilkins, 1996), which is the large-scale analysis of many proteins simultaneously (David *et al*., 2005b; Hoerndli *et al*., 2005). While this could potentially include all the proteins encoded by the genome at any given time (i.e. the proteome), it more commonly refers to specific subsets of proteins found in specific regions or cell-type, under specific conditions (Pandey & Mann, 2000; Righetti *et al*., 2004). Sensitive proteomics techniques have been developed to determine the structure, localization, biochemical activity, interactions (i.e. protein-protein, protein-lipid) and the cellular roles played by a multitude of proteins, with the results translated either to a natural physiological or to a pathological state of events (Onn & Mann, 2005).

Substantial technological advances have been made in novel instrumentation, experimental strategies, and bioinformatics tools for analyzing the proteome (De Hoog & Mann, 2004).

However, despite the bewildering range of available techniques and analysis tools, some form of protein separation procedure, followed by techniques for peptide detection and identification are routinely required, with mass spectrometry (MassSpect) remaining its key technology (Fig. 1). The speed of advancements in peptide-sequencing technology may allow quantifying most cellular proteins by high resolution MassSpect (Lipton *et al*., 2002). To quantify proteins according to their degree of expression, MassSpect technology is frequently applied in conjunction with two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). This combined experimental approach allows for inter-sample comparisons relating to the disease state as well as between experimental subjects and normal controls (De Iuliis *et al*., 2005).

There is an inherent risk of preferentially detecting proteins that are present in abnormally high concentration, thereby repressing the weaker signals by less abundant proteins (Fountoulakis, 2001; Fountoulakis, 2004; Vercauteren *et al*., 2004; Wiederkehr, 1991). To reduce the complexity of the protein sample, and thereby increase sample resolution, strategies have been developed to reduce levels of highly abundant (and generally less interesting) proteins, which may obscure detection of the less abundant ones. In blood-based proteomics, this can be achieved by affinity separation of abundant proteins (such as albumin, fibrinogen and transferrin) from either human or mouse plasma, using affinity-purified antibodies covalently conjugated to microbeads. Another practical and economically feasible approach is to first apply proteomic techniques to detect for markers of the disease in organs where protein concentrations are expectedly high, before searching for them in fluids where they are less abundant (Meyer & Stuhler, 2007). The application of this approach to neurodegeneration is not trivial, as human brain tissue can be of bad quality and show high variability, e.g. with regards to gender, age and medication of patients. Nonetheless, one may be able to choose tissue from brain areas that are affected only relatively late in disease and thus spared from massive degeneration. For Alzheimer's disease (AD), this could be the brain stem as a brain area that is relatively preserved in late stage AD, but still displays the typical hallmark lesions of the disease. Alternatively, animal models of AD or PD may be employed to apply proteomics first to dissected brain areas and eventually body fluids such as plasma or CSF, which are likely to reflect, at least in parts, the deregulation of proteins in the brain (Götz *et al*., 2007). Subcellular proteomics provides another means for combining the direct analysis of the proteome of isolated organelles such as mitochondria. Methods for fractionating subcellular organelles include traditional techniques, such as centrifugation (e.g. high-speed sedimentation/density gradient) (Pasquali *et al*., 1997; Fialka *et al*., 1997; Tribl *et al*., 2006a; Tribl *et al*., 2006b), but also highly sensitive commercial fractionation kits for extracting nuclear, cytosolic, mitochondrial or membrane fractions (Huber *et al*., 2003; Heimlick & Cidlowski, 2006; Chen *et al*., 2005b; Vila-Carriles *et al*., 2007). The limitations of the subfractionation approach are in its experimental

variability and the fact that the fractions are rarely pure as, e.g. in synaptosomal preparations, mitochondria are easily co-purified. The subcellular fractionation and purification techniques can be effectively combined with gel-dependent methods, such as 2-D PAGE, or gel-independent ones.

In 2-D PAGE, proteins are first separated in the first dimension according to their charge (z) and molecular weight using isoelectric focusing (IEF) (Klose, 1975; O'Farrell, 1975; Görg *et al*., 1985) (Fig. 1). The proteins migrate through a thin gel-strip embedded with immobilized pH gradients (IPG). Migration terminates when the protein reaches the point where the net charge is neutral (i.e. the isoelectric point, or pI). Different IPG strips can be used: wide pH ranges (IPG 3-10) that cover many pH units, those that cover narrower ranges (IPG 4-7), or very narrow ones that only provide a single pH unit-width (IPG 5-6, or IPG 5.5-6.7) (Simpson, 2003). Narrow pH ranges allow zooming in and give a better resolution of the protein spot pattern within a smaller window (Hoving *et al*., 2002). Proteins are then separated in the second dimension (orthogonal direction) using electrophoresis that takes place in acrylamide gels containing sodium dodecyl sulphate (SDS), where SDS imparts a net negative charge, allowing proteins to separate according to mass (m). Separation in these two dimensions allows for the resolution of multiple isoforms and variants of the same protein. A variety of chemical stains and fluorescent markers are available to visualize and detect the differences in protein staining intensities between samples (David *et al*., 2005a). Approximately 1000-3000 proteins can be visualized per stained gel, with the capacity to resolve up to 10,000 proteins in a single gel (Klose, 1975; O'Farrell, 1975). A comparison of different 2-D PAGE methods and their application to mouse brain has been done by several groups including Stühler and colleagues (2006).

Despite 2-D PAGE being a relatively old-fashioned biotechnological technique, from a proteomics perspective it remains popular since it allows for the use of high through-put screening in an easily executable and cost-effective manner. However, its main drawbacks are the limited dynamic range and often a failure of detecting membraneous or low abundant proteins (Aebersold *et al*., 2000). In addition, it is often difficult to accurately compare two samples when the comparison is based on the degree of protein-expression alone, since protein spots often overlap or appear small and/or blurry, thereby increasing the risk of over- or undercounting them.

The technique known as ‘Differential In-Gel Electrophoresis’ (DIGE) by which the samples are labeled with different fluorescent dyes (such as Cy3 and Cy5) and then separated on the same gel, has proved very useful in increasing the dynamic range and reducing technical variability (Wu, 2006).

Liquid chromatography (LC) coupled to MS has proved to be an important alternative to 2-D gels (Fig. 1). Various LC techniques (such as affinity, size exclusion, reverse phase or charge

chromatography) provide different means for separating complex samples (Wolters *et al*., 2001). The separated protein mixture is then fed directly into an electrospray ionisation (ESI) MS, using tubes of microcapillary diameter, which greatly reduces the amount of sample needed for analysis (Graves & Haystead, 2002; Link *et al*., 1999; Opiteck *et al*., 1997; Washburn & Yates, 2000). The LC-MS technique is particularly useful for membrane-associated proteins, phosphopeptides, enzymes such as kinases and phosphatases, and transcription factors that are difficult to detect through 2-D PAGE because of their low levels of abundance (Washburn *et al*., 2002). Of the various ionization methods developed for coupling, LC to MS-ESI is the most widely used technique that allows for the gentle ionization of large biomolecules (Jorge *et al*., 2007). An alternative technique is MALDI (Matrix-assisted laser desorption/ionization), a "soft" ionization technique used for analyzing biomolecules and large organic molecules that tend to be too tender to be fragmented and ionized by other, more conventional means. Compared to MALDI, ESI takes longer to perform but has the advantage of easy coupling to separation techniques such as LC or HPLC. This allows for high-throughput and on-line analysis of peptide and protein mixtures.

An ionization method known as SELDI (surface-enhanced laser desorption/ionisation) makes use of the protein-chip array technology (Hutchens & Yip, 1993). The "chip" consists of a thin strip of aluminium containing eight sample-loading areas. Depending on the nature of the chip surface, only specific types of proteins bind. In other words, the chip forms the target surface to which proteins and matrices are applied. Depending on the composition of the matrix, particular types of proteins (acidic, basic, antigens, etc.) are captured. For example, an IMAC30 ProteinChip Array that is activated by coating the surface with transition metals such as gallium allows for the detection of posttranslational modifications such as phosphorylation (Escher *et al*., 2007). This method is typically used in combination with time-of-flight MS (TOF-MS) and is similar to MALDI. The speed and ease of SELDI-TOF in principal allows for use in a clinical environment (Paweletz *et al*., 2001), but compared to other MS methods, SELDI does not allow for the identification of peptides or proteins but only detects signals.

Whereas some techniques have been developed with the aim of providing qualitative information on protein expression, a number of methods have been developed to specifically obtain quantitative information. One such method is the ICAT (isotope coded affinity tag) technique, where cysteine residues are differentially tagged with stable isotopes (Olsen *et al*., 2004). Proteins from two different samples (e.g. control and experiment) are labeled with either deuterium d0 (light) and d8 (heavy) tagging reagents, which have a mass difference of only eight mass units. This allows for mixing two samples prior to separating them on the same gel, thereby eliminating in-between-gel variation. A comparison of the peak intensities of these tags provides information on the differential expression of the proteins of interest under two different

conditions. ICAT is particularly well suited for targeting membrane proteins, as it is compatible with strong detergents (Gygi *et al*., 1999; Moseley, 2001). However, drawbacks of the ICAT method are that it requires the presence of a cysteine in the protein sequence and that it usually requires large initial sample sizes, in the order of 100 µg. Other labeling methods were recently developed to accommodate smaller sample sizes, such as labelling with ^{12}C and ^{13}C, or ^{16}O and ^{18}O (Zang *et al*., 2004).

iTRAQ (Isobaric Tagging for Relative and Absolute protein Quantification) is another quantitative protein identification approach (Fig. 1). It was the first reagent developed for LC-based differential proteome analysis. The isobaric tagging reagents consist of a reporter group, a balance group (to make up for the mass difference of the reporter group), and a peptide reactive group. The latter covalently links an iTRAQ tag with each lysine side chain and amino-terminal group of a peptide, allowing for labeling multiple peptides in a sample digest. The complex mixtures are then fractioned by chromatographic means and analyzed with LC/MS/MS (Olsen *et al*., 2004, Abdi *et al*., 2006). Together with ICPL (isotope coded protein label) (Schmidt *et al*., 2005), iTRAQ has now more or less substituted ICAT. In biomarker research, however, label-free MS is increasingly applied, owing to the high costs of ICAT or iTRAQ labeling, since large numbers of samples need to be analyzed (Horvatovich *et al*., 2007).

MECT (metal element chelated tags) uses eight uncommon earth metals that couples to peptides. It is cheaper than isotope-based methods, with the bicyclic anhydride diethylenetriamine-N,N,N',N' ',N' '-pentacetic acid (DTPA) coupling covalently to primary amines of peptides, and the ligand then chelating to the rare earth metals Y and Tb. The tagged peptides are mixed, analyzed by LC-ESI-MS/MS, and peptides quantified by measuring the relative signal intensities for the Y and Tb tag pairs (Liu *et al*., 2006).

Strategies are constantly improved to functionally categorize the stream of proteins that are newly discovered through proteomics. One technique that was recently shown to be effective for establishing cofactor preferences for proteins is fingerprinting with saturation transfer difference (STD) NMR for detecting closely related cofactors. This approach was validated with both dehydrogenases and cyclic nucleotide-binding proteins (Yao & Sem, 2005).

The increased use of proteomics in the field of neurodegeneration research is greatly assisted by the development of integrated technologies that apply high-resolution separation strategies to complex protein samples, combined with sophisticated computer-based identification methods. Ingenious novel hardware and software developments continuously lower detection limits, while widening the dynamic range, making profiling of highly complex protein mixtures for identification of PD biomarkers possible. Although the different search engines and databases (i.e. SwissProt, MASCOT) vary in their sensitivity and accuracy for detecting protein sequences, eventually this should have little influence on experimental

outcomes, due to the standardization in data collection and interpretation that is enforced by the proteomics community.

3. PROTEIN MARKERS FOR MITOCHONDRIAL DYSFUNCTION AND OXIDATIVE STRESS IN PD

Mitochondrial dysfunction and oxidative stress have been implicated in PD (Schapira, 1998; Betarbet *et al*., 2000; Hardy *et al*., 2003; Kaur *et al*., 2003; Lopes & Melov, 2002; Beal, 2003). Proteomics has proved to be a useful tool to examine the oxidative-stress induced changes in proteins (Butterfield & Castegna, 2003). A deficit of complex I of the respiratory chain has been shown by reduced activity of the enzyme NADH-ubiquinone reductase in neuronal tissue, such as the SN (Schapira *et al*., 1989; Dexter *et al*., 1994) and frontal cortex of PD patients (Parker *et al*., 2008), as well as in non-neuronal tissue such as blood platelets (Parker *et al*., 1989; Benecke *et al*., 1993; Haas *et al*., 1995; Swerdlow *et al*., 1996; Gu *et al*., 1998) and skeletal muscle (Bindoff *et al*., 1991, Shoffner *et al*., 1991). A proteomic analysis of PD and control SN revealed that neurofilament chains were less abundant in PD specimens, whereas peroxiredoxin II, mitochondrial complex III, and the ATP synthase D chain, among others, were significantly increased in PD samples compared to controls, indicating a compensatory upregulation in surviving SN neurons (Basso *et al*., 2004). A role for mitochondrial dysfunction in PD is further supported in animal models as administration of environmental toxins known to inhibit mitochondrial function caused dopaminergic cell loss (Betarbet *et al*., 2000; Di Monte *et al*., 2000) and increased production of ROS, another correlate of the clinical phenotype (Beal, 2004).

Ubiquitin carboxy-terminal hydroxylase L-1 (UCHL1), also known as PGP9.5, is one of the most abundant neuronal proteins present in brain (Wilkinson *et al*., 1989). Studies have shown that it is extensively downregulated and structurally modified in the presence of oxidative stress (Castegna *et al*., 2002; Choi *et al*., 2004). In addition, mutating isoleucine 93 to methionine of UCHL1 causes autosomal dominant PD (Leroy *et al*., 1998). Furthermore, increased amounts of oxidatively modified UCH-L1 were found in sporadic PD cases compared to normal brain (Castegna *et al*., 2002; Choi *et al*., 2004; Butterfield *et al*., 2006). This deregulation has been implicated in the pathogenesis of PD since oxidative modification and the subsequent decrease in UCH-L1 enzymatic activity may affect normal neuronal function and plasticity (Setsuie & Wada, 2007). Interestingly, increased expression of UCH-L1 has been found also in AD using MALDI MS (Sultana *et al*., 2007). Hence, the application of redox proteomics may be an invaluable tool to help ascertain the role played by oxidised UCH-L1 and other proteins in the pathogenesis of PD and possibly, AD.

MPTP is a potent mitochondrial toxin that causes dopaminergic degeneration and LB formation in nigral neurons. When mice were chronically treated with MPTP, compared to control animals, more than 300 mitochondrial-related proteins were identified, of which more than 100 displayed a differential expression pattern (Jin *et al*., 2005). A category analysis revealed their involvement in signal transduction, the ubiquitin-protease system, oxidative stress and mitochondrial function, all of which has been implicated in the pathogenesis of PD (Dauer & Przedborski, 2003; Fiskum *et al*., 2003; Rego & Oliveira, 2003). Of the more than 300 proteins detected, DJ-1 was validated by Western blotting.

As discussed before, genetic mutations have been identified in a small subset of cases, mainly in families with early-onset familial PD. DJ-1 is one of the genes implicated in disease pathogenesis (Bonifati *et al*., 2003). DJ-1 has also been identified as a possible marker in plasma for the progression of sporadic PD (Waragai *et al*., 2007). Gene-based animal models indicate that DJ-1-associated disease onset and progression may operate through the ubiquitin-proteasomal pathway, which is a molecular pathway common to all genetic variants of PD (Dauer & Przedborski, 2003; von Bohlen und Halbach *et al*., 2004).

In its mutant form, DJ-1 confers a reduced protective function against endoplasmic reticulum stress. Two recent reports (Taira *et al*., 2004; Yokota *et al*., 2003) show that DJ-1 has a repressive effect on cellular toxicity induced by overexpression of Pael-R, a newly identified substrate for parkin that is localized within the core of LBs (Yokota *et al*., 2003). Pael-R has also been assigned a distinct role in LB formation (Murakami *et al*., 2004).

DJ-1 seems to antagonize many of the deleterious effects of SNCA, including its aggregation (Shendelman *et al*., 2004; Zhou & Freed, 2005; Zhou *et al*., 2006). Therefore, although the exact physiological function of DJ-1 remains elusive, increasing evidence suggests that it may confer protection, for instance as an anti-oxidative protein. In this regard, DJ-1 has been identified as a hydroperoxide-responsive protein that increases its acidity following exposure to oxidative stress (Mitsumoto & Nakagawa, 2001; Mitsumoto *et al*., 2001), thereby revealing the presence of oxidative stress (Kinumi *et al*., 2004; Taira *et al*., 2004).

In addition to this role, many lines of evidence argue that DJ-1 modulates the function of SNCA and vice versa (Zhou *et al*., 2004; Jin *et al*., 2005). Robust proteomics, consisting of stable isotope labelling by amino acids in cell culture (SILAC) was used on dopaminergic neurons exposed to the mitochondrial toxin rotenone (Jin *et al*., 2007). Compared to controls, 324 proteins were associated with SNCA and 306 with DJ-1. Although no direct interaction has been observed between DJ-1 and SNCA, 144 proteins mutually associated with DJ-1 and SNCA, displaying significant changes in terms of relative abundance. Further validation was performed on a subset of these, revealing that the docking proteins mortalin (mthsp70/GRP75), nucleolin, grp94, calnexin and clathrin associate with both SNCA and DJ-1. The study

confirmed the absence of a direct interaction between SNCA and DJ-1 and reported the identification of five novel proteins that associate with both SNCA and DJ-1. It is anticipated that future work will shed light on the functional interaction between these two critical proteins and the mechanism by which they participate in cellular functions and PD pathogenesis.

Mitochondria-enriched fractions obtained from the SN of mice chronically treated with MPTP have been subjected to ICAT and compared with controls (Jin *et al*., 2005). The animals received a 5-week treatment regime together with an adjuvant, probenecid (prob) that caused selective nigrostriatal neurodegeneration and the formation of LB-like cytoplasmic inclusions in surviving nigral neurons. After identifying more than 300 proteins, of which >100 displayed changes in relative abundance between the MPTP/prob-treated mice and controls, the high throughput proteomic analysis was validated by Western blotting. This demonstrated that one of the identified proteins, DJ-1, co-localized with SNCA in dopaminergic neurons as well as in cytoplasmic inclusions in the mice that had been treated with MPTP/prob. By using this particular proteomics approach, the study successfully overcame a limitation usually imposed when using pooled samples that prevents the results from telling whether the DJ-1 increase results from a single mouse or from multiple mice. By conducting Western blot analyses in separate animals a DJ-1 increase was demonstrated in the majority of subjects.

Jin et al. (2007) created a quantitative profile of mitochondrial proteins harvested from PD patients compared to normal age-matched controls. In addition, they used a cellular model of PD, consisting of rotenone-treated dopaminergic cells. In this study, a 'shotgun proteomic' approach was used called multidimensional protein identification technology (MudPIT), that entails the direct analysis of complex samples for rapidly producing a global profile of the protein complement (Link *et al*., 1999; Wu & MacCoss, 2002). To quantify the proteins, ICAT was combined with SILAC (Jin *et al*., 2007). Among the differentially expressed proteins was mortalin, a mitochondrial stress protein that was substantially decreased in both PD brain and the cellular PD model. The study further showed that several mortalin-binding proteins might participate in rotenone-mediated toxicity and that by overexpressing and/or silencing mortalin expression, PD-related pathology was significantly affected. Together, the studies suggest that mortalin modulates PD development via pathways involving mitochondrial and proteasomal functions as well as oxidative stress (Jin *et al*., 2007).

By integrating proteomics technologies (2-D gel electrophoresis and MALDI-TOF), Lee et al. (2003) gathered evidence that expression levels of calreticulin, a high-capacity calcium buffering protein located in MN9D dopaminergic cells may correlate with the degree of cell death following 6-OHDA treatment. This implies that calreticulin may either directly mediate 6-OHDA-induced cell death or, alternatively, it may participate in inducing a general cellular response prior to apoptosis.

Calreticulin further functions as a chaperone by binding to oligosaccharide moieties on proteins in addition to retaining proteins in the ER (Spiro *et al*., 1996). Its other assigned roles have been controversial, such as promoting cell death when over-expressed (Arnaudeau *et al*., 2002; Nakamura *et al*., 2000), as other studies suggest that it might be involved in preventing oxidative stress-induced cell death (Liu *et al*., 2001; Nunez *et al*., 2001). Although the direct mechanisms through which calreticulin might induce dopaminergic neuronal death remain to be established, proteomics suggests that this protein may be a marker of oxidative stress (Lee *et al*., 2003).

Parkin is another marker for oxidative stress and mitochondrial dysfunction in PD. Periquet et al. (2005) generated parkin knock-out mice and searched for possible changes in the brain proteome using 2-D-DIGE. The study indicated compensatory mechanisms that protect dopaminergic neurons from ROS-induced death. This phenomenon was ascribed to 87 proteins that differed in relative abundance compared to wild-type brains. A high proportion of the altered proteins related to energy metabolism, detoxification, stress-related chaperones and components of the ubiquitin-proteasome pathway. In a related study, parkin knock-out mice were analyzed by 2D-PAGE followed by MS (Palacino *et al*., 2004). This revealed a decreased abundance of a number of proteins involved in mitochondrial function or oxidative stress that could be confirmed functionally. The mice also showed decreased serum antioxidant capacity and increased protein and lipid peroxidation. Together, this identifies an essential role for parkin in the regulation of mitochondrial function. Interestingly, in the mice mitochondrial dysfunction and oxidative damage was found in the absence of nigral degeneration (Palacino *et al*., 2004). This is supported by studies in parkin-deficient flies (Greene *et al*., 2003) and reminiscent of P301L tau mutant mice that model aspects of AD and also show mitochondrial dysfunction and oxidative damage, in the absence of overt cell loss (David *et al*., 2005a; David *et al*., 2006; Götz *et al*., 2008).

Another protein found in an oxidatively modified state in PD brain tissue is superoxide dismutase 1 (SOD1) that was identified by MALDI-TOF MS in a cell-culture system in a screen for changes in mitochondrial protein expression (Wood-Allum *et al*., 2006). In PD, not only is residue Cys-146 of SOD oxidized to cysteic acid, but SOD1 is also carbonylated (Choi *et al*., 2005). The nature of these modifications are still unclear, and it is hoped that proteomics will provide an explanation for the accumulation and posttranslational modifications of SOD1 in the PD brain (Bandopadhyay *et al*., 2004).

4. ALPHA-SYNUCLEIN AND ITS ROLE IN PD

Alpha-synuclein (SNCA) is a presynaptic protein associated with PD pathogenesis. It appears in both a soluble and membrane-bound form (Zhu *et al*., 2006a), and comprises the central

filamentous component of LBs. Under physiological conditions, it forms a protein complex in the cytosol. As interacting proteins in this complex, 250 candidate proteins have been identified by Zhou and coworkers (2004). However, DJ-1 was not among them. It is important to note, that a 'negative' proteomic result does not necessarily entail that the protein of interest is completely absent within the protein complex (Corthals *et al*., 2000), especially when immunohistochemistry has been applied. For example, Neumann et al. (2004) and Jin et al. (2007) convincingly demonstrated that DJ-1 is present within the halo part of LBs that is known to contain insoluble SNCA.

In an effort to identify proteins that are associated with SNCA and may contribute towards its aggregation, Zhou et al. (2004) exposed MES rat cells to rotenone. Duplicate 2-D gels were run with one gel probed with an anti-SNCA antibody, while the other was stained with Coomassie Blue. The stained gel sections that corresponded to SNCA-immunoreactive bands were cut, trypsin-digested and analyzed with LC-MS/MS spectrometry. The study identified more than 250 proteins associated with SNCA. Under conditions where rotenone was cytotoxic and induced the formation of SNCA-immunoreactive cytoplasmic inclusions, >51 proteins displayed significant differences in terms of relative abundance. This list included heat shock proteins (HSP) 70 and 90 which, when overexpressed in MES cells not only protected cells from rotenone-mediated cytotoxicity, but also decreased SNCA aggregation. From this, it can be concluded that the protection afforded by hsp70 transfection may relate to suppression by rotenone-induced oxidative stress (Zhou *et al*., 2004). This putative protective role of hsp70 was further supported in subsequent in vivo studies carried out by Klucken et al. (2004). Other neuronal proteins that may couple to SNCA in a similar fashion include parkin (Paciello *et al*., 2006) and the microtubule-associated protein tau (Jensen *et al*., 1999), the principal component of the neurofibrillary tangles in AD (Chen *et al*., 2004).

A 'shotgun proteomic' experimental approach was applied to A30P SNCA transgenic Drosophila and age-matched controls (Xun *et al*., 2007). By conducting the analysis at seven different time-points across the organism's adult lifespan, disease-associated differences appeared to change substantially as the flies aged. Interestingly, the majority of the perturbed protein levels only existed over a narrow distribution of ages indicating the necessity to take multiple time-points for analysis.

5. ROLE OF THE CYTOSKELETON AND SMALL PEPTIDES IN PD

Axonal transport and in particular the integrity of microtubules is essential for neuronal function. Not surprisingly, impaired axonal transport has been implicated in a range of neurodegenerative disorders (Götz *et al*., 2004). Among the proteins with a causal role in AD is tau, a microtubule-associated protein, that similar to SNCA, is found in an unfolded state when in solution. Tau is

mainly a neuronal protein. However, it has been found also, although at low levels, in astrocytes and oligodendrocytes (Chen *et al*., 2004; Götz, 2001; Davidsson & Sjögren, 2005). Tau's cardinal functions in neurons are to regulate the assembly and stabilization of microtubules that determine cellular morphology and serve as tracks for vesicles, axonal proteins and mitochondria (Shahani & Brandt, 2002). Under physiological conditions, tau is transported along axons at a rate compatible with a slow transportation mechanism (Mercken *et al*., 1995; Utton *et al*., 2002; Zhang *et al*., 2004). Under pathologic conditions such as AD and frontotemporal dementia (FTD), tau aggregates and form filaments, which fill up the entire soma of a degenerating neuron. Here, a failure of axonal transport has been implicated as a possible mechanism underlying tau accumulation (Praprotnik *et a*l., 1996; Flament-Durand & Couck, 1979; Richard *et al*., 1989; Bendiske *et al*., 2002; Dai *et al*., 2002; Götz *et al*., 2006).

There is increasing evidence for an overlap between PD and AD, not only in clinical terms (Kurosinski *et al*., 2002), but also biochemically and histopathologically (Hoerndli *et al*., 2005). For example, LBs are not only present in PD brain, but also abundant in Dementia with Lewy bodies (DLB) and in the LB variant of AD (Hansen *et al*., 1990). Specifically, LBs have been detected in the amygdala of more than half of all familial AD cases, and some LBs colocalized with tau-positive neurofibrillary tangles (NFTs) (Lippa *et al*., 1998). Biochemically, many of the functional categories, which are deregulated in PD are also deregulated in AD (Hoerndli *et al*., 2005).

Another cytoskeletal protein with a role in neurodegeneration is β–actin, found abundantly within neurons, astrocytes, and blood vessels in the CNS, and concentrated particularly along the periphery of the neuronal perikaryon (De Iuliis *et al*., 2005). This preferred location places it in an ideal position to integrate incoming signals and produce the mechanical tension necessary for shaping the perikaryal surface (Pannese *et al*., 1996). A recent study found that specific protein carbonyl levels in β–actin are significantly higher in postmortem human AD brain samples than in controls (Aksenov *et al*., 2001). Also, when a 'hemiparkinson' rat model of PD was obtained by unilaterally injecting the neurotoxin 6-OHDA, De Iuliis et al. (2005) found increased levels of β–actin in the ipsilateral striatum, compared to the contralateral side (Lee *et al*., 2003). Previous studies described β-actin to be in an oxidized state in AD again suggesting common patho-mechanisms for the two diseases.

Much of the signaling in brain is mediated by small peptides. The study of endogenous peptides, termed peptidomics, however is complicated by the presence of protein fragments produced post-mortem during conventional sample handling. This can be partially prevented by rapidly heat-denaturing brain tissue before analysis (Svensson *et al*., 2007). This approach has been applied to the MPTP model for PD, by which a polypeptide termed PEP-19 (Peptide 19) was found to be significantly decreased in the striatum of treated animals compared to controls

(Svensson *et al*., 2007). PEP 19 is a 7.6-kDa polypeptide that binds calmodulin and prevents calcium-calmodulin-dependent signaling (Ichikawa & Sugimoto, 2005). As a neuron-specific calmodulin-binding polypeptide, it is believed to play a pivotal role within the second messenger system that allows for the transduction of signals within cells. Elevated levels have been found in selective neuronal populations, such as the granule cells of the hippocampus and the Purkinje cells of the cerebellum, implying resistance towards degeneration (Johanson *et al*., 2000). In contrast, Skold et al. (2006) recently demonstrated by in situ hybridization analysis, that PEP-19 mRNA levels are significantly decreased in the striatum, following MPTP administration in mice. Disease-specific differences have been found in an earlier study (Utal *et al*., 1998) that reported that although PEP-19 immunoreactivity appeared to be significantly reduced in AD and Huntington's disease brain, no apparent alterations were detected in PD brain. Thus, the exact nature of PEP-19 in PD remains controversial.

6. FROM POST MORTEM TISSUE TO BODY FLUIDS AS SOURCES OF PROTEINS

Proteome searches are frequently conducted within post-mortem brain tissue of a diseased state. This approach entails its own set of difficulties, including the potential confounds caused by additional age-related diseases that the usually aged patient may have suffered from, the time between death and autopsy that could have resulted in protein changes unrelated to the disease, how rapidly death ensued, the time of death and the duration of the coma that preceded death. In addition, the pharmacological treatment, i.e. the medication, may result in deranged protein levels, that may be mistaken for being disease-related. It is essential to consider the homogeneity of all these parameters in both the control and the patient group (Riederer & Wuketich, 1976). However, in spite of these difficulties several groups have proteomically probed brain tissue for parameters that may assist in predicting or diagnosing the disease, monitor its progression, or be used to follow-up therapy outcomes (Hoerndli *et al*., 2005).

In addition to post-mortem tissue, body fluids such as cerebrospinal fluid (CSF), serum, blood or urine are increasingly collected from either animal models or humans and subjected to proteomics analysis. Obviously, animal models do not reproduce all aspects of disease, especially as far as the clinical features and the spatiotemporal distribution of brain lesions is concerned, but at a single cell level and biochemically, they have proved to be a very powerful tool (Götz *et al*., 2004).

Although there is currently no blood test available with which to confirm the diagnosis or monitor PD, the accessibility of serum, as a component of blood, that is more readily available than any other body-fluid, offers potential exploitation using proteomics technology. Sophisticated techniques, including MS are used to identify rare proteins in blood serum and

CSF to identify biomarkers that represent unique proteomes. At present, thirty-four blood serum protein biomarkers are used for diagnosing neurodegenerative diseases (Sheta *et al*., 2006).

In particular, CSF has been used as a medium from which to launch a proteome-based search strategy for detecting disease biomarkers, since the CSF reflects the state of proteins present in the brain under healthy and diseased conditions (Hühmer *et al*., 2006). As a complex mixture, consisting of proteins, peptides, proteolytic fragments and antibodies, CSF provides an excellent repository of pathologic information concerning the CNS (Romeo *et al*., 2005).

In one study, quantitative proteomics has been applied successfully to CSF samples from patients with PD at different stages as well as with AD and compared to normal controls (Pan *et al*., 2008). The frequently encountered problem was addressed that while biomarkers are generally validated by enzyme-linked immunosorbent assays (ELISAs), often there are no specific antibodies available to set up these assays. Hence Pan and coworkers (2008) used a quantitative LC-MALDI TOF/TOF approach by spiking CSF samples with isotope-labeled peptides derived from a total of 14 proteins that had been previously identified as being deregulated in disease. Importantly, they were able to identify and quantify the peptides in CSF without prior depletion of abundant proteins (Pan *et al*., 2008). It should be mentioned that for quantitative proteomics of body fluids, label-free profiling has been successfully used by applying a microfluidics-based chip-LC-MS system, a method that has gained increased attraction for reasons of cost (Horvatovich *et al*., 2007). Sophisticated softwares have been developed to assist in the quantification of label-free profiling, including the open software tool, SuperHirn (Mueller *et al*., 2007).

Several strategies exist for characterizing lipid-metabolizing proteins in human CSF. These proteins are potentially therapeutic targets due to their ability to transport lipids required for neural growth or to convert these into molecules that control brain physiology (Fonteh *et al*., 2006). By combining lipid analysis with proteomics, the existing knowledge of disease pathology may be enhanced and the likelihood increased for discovering distinct markers and biochemical mechanisms of disease. In an effort to profile tau in CSF as a reflection of the degree of neuronal degeneration and damage sustained, Blennow et al. (1995) combined a pre-fractionation step consisting of liquid phase isoelectric focusing (LP-IEF) with immunoblotting. This revealed that both phosphorylated and unphosphorylated forms of tau are present in CSF and that tau appeared both in truncated and full-length versions. For a comprehensive review of the posttranslational modifications of tau, the reader is referred to Chen et al. (2004).

Quantitative 2-D gel electrophoresis has been used to analyze serum proteins derived from 422 patients suffering from different neurodegenerative diseases and compared with normal controls, in an effort to identify potential biomarkers (Goldknopf *et al*., 2006). Differential protein spots were found for a total of 34 serum proteins between amyotrophic lateral sclerosis (ALS),

PD and related disorders, with nine relating to the complement system. Components of complement C3, including C3c and C3dg as well as complement factor H showed a marked increase in both disease-types compared to controls. In addition, the results indicated elevated levels for full-length factor B for PD, but not the control group. This reported elevation of factors H and B contradicts recent work proclaiming that they are significantly reduced in PD CSF (Finehout *et al*., 2005). In this study, it is possible that elevations of fragment Bb arose from elevated levels of factor H, which has been found to induce dissociation of C3 convertase ($C3bBb(Mg^{2+})$)(Hourcade *et al*., 2002), thereby liberating factor Bb and interrupting the alternative pathway of the complement cascade (Goldknopf *et al*., 2006). The study generated a list of potentially useful biomarkers for further exploitation in future studies. In addition, the identified proteins provide evidence for an involvement of neuro-inflammatory processes in the pathogenesis of ALS and PD. The study indicates further that a comparison of the expression level of individual protein isoforms in conjunction with a measurement of the total protein expression level may be useful for investigating a protein as a possible biomarker.

The results on the complement cascade add strength to existing evidence suggesting that inflammatory processes involving complement activation may play a definitive role in the onset of PD. It also complements work that provides evidence for neuronal injury resulting from activated microglial release of free radicals in PD (He *et al*., 2002). This finding provides a potential avenue for developing treatment targets and for monitoring their clinical application. It further shows that two different neurodegenerative diseases can differentially express members of the complement system. In another study performed by Sjögren et al. (2001) it was found that the CSF concentrations of tau and phosphotau are increased in around two-thirds of probable AD cases while revealing normal levels in PD and in controls. The insight gained from such studies may potentially provide valuable information regarding their respective pathological mechanisms and identify markers that are shared between diseases and others which are disease-specific. In these endeavours, it is worth mentioning that the analysis of post-translational modifications remains a technological challenge, but various generic strategies have recently emerged to aid this strategy (Reinders *et al*., 2004; Fountoulakis & Kossida, 2006).

7. FUTURE DIRECTIONS

It is evident that our present knowledge of PD pathogenesis and potential treatment strategies is inadequate. A growing body of evidence indicates that the accumulation of altered proteins and impaired protein clearance may be a common pathomechanism in both familial and sporadic PD. To fully understand the role played by abnormal protein aggregates in PD, it is imperative that additional proteins with a role in disease are identified and that their disease-

related roles are defined. Recent advances in proteomics indicate that this technology may equip us with methodologies with which to study PD in a systematic manner that has not been possible to this extent in the past, by offering the opportunity to explore the proteome at all levels, ranging from fundamental neuroscience to clinical trials. However, to utilise neuroproteomics to its full potential, it is imperative that standard operating protocols are developed that allow these techniques to be applied reproducibly in a clinical setting.

The systems-based approach offered by proteomics not only allows for identifying multiple key proteins and signaling cascades, but also shows how these proteins interact with each other. It is anticipated that such data may eventually improve our understanding of the disease at a biochemical level and help identify novel biomarkers to assist in making an early, presymptomatic diagnosis of PD. In addition, it is hoped that the application of this technology will lead to the identification of new targets for more effective therapeutic intervention. The clinico-pathological profiles of AD and PD seem to overlap, or at least synergise to a certain extent (Kurosinski *et al*., 2002). Information is increasingly emerging to support such a postulation and therefore, the possibility is increasingly upheld that drugs that target the blocking of SNCA or tau may therapeutically benefit a much broader spectrum of neurodegenerative disorders.

Since PD likely has a multifactorial etiology, proteomics provides the possibility for characterizing inter-patient variance. The lessons learned from this may eventually assist in developing personalized therapies, resting on the basis of individual patterns of protein expression (Jain, 2004). With this in mind, the future development of protein markers with which to identify not only the motor-related, but also the non-motor manifestations of PD, including cognitive dysfunction, autonomic dysfunction and speech disturbance, will contribute greatly towards a more global perspective of the disease.

The greatest challenge facing the optimal utilization of this technology lies in detecting and quantifying low-abundant and hydrophobic proteins. In addition, the accurate detection of post-translational modifications, their origin and the role they play in PD should also be made a priority. However, it is anticipated that protein chips and miniature separation systems will play a significant role in overcoming these limitations.

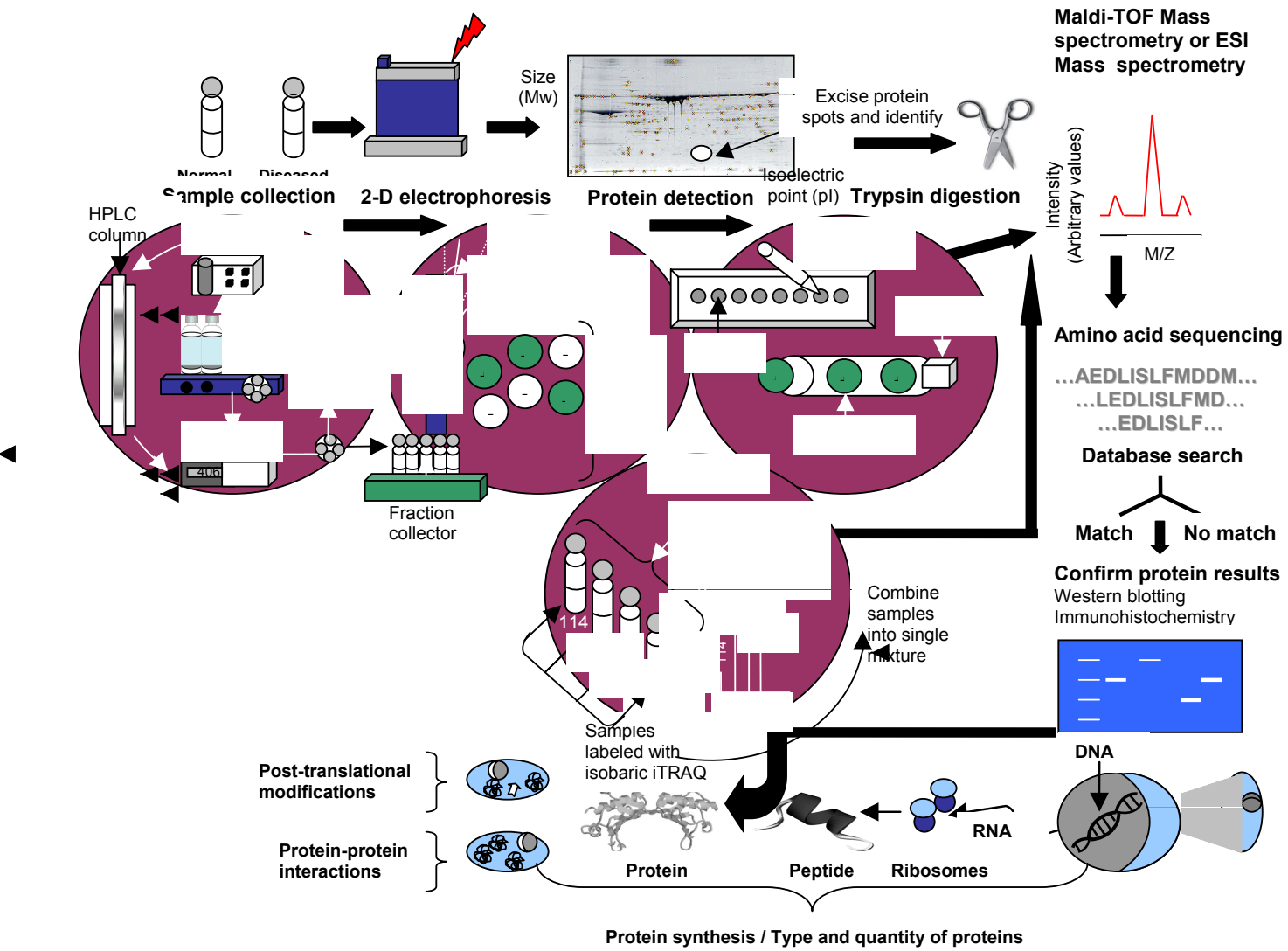

Fig. 1. A flow-diagram illustrating the sequence of steps involved in sample analysis using the SELDI, MALDI, LC-MS and iTRAQ proteomic strategies. As illustrated, these methods provide information on the type and quantity of proteins, post-translational modifications and protein-protein interactions. Figure taken from Pienaar *et al.* (2008).

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CHAPTER 7

A PROTEOMICS AND MOTOR-FUNCTION ANALYSIS OF THE EFFECTS OF SIMVASTATIN ON MITOCHONDRIAL PROTEINS IN A ROTENONE RAT MODEL OF PARKINSON'S DISEASE

1. INTRODUCTION

Accumulating evidence suggests that the etiology of the neuronal loss seen in the non-familial forms of PD may involve several molecular and cellular events, including oxidative stress, the accumulation of altered proteins, excitotoxicity, proapoptotic mechanisms and dysregulated mitochondrial functions (Dauer & Przedborski, 2003; Hirsch *et al*., 2005). This was further substantiated by reports that the effects of various chemically-induced parkinsonian models, including MPTP, rotenone, paraquat and most recently, epoxomycin are primarily due to mitochondrial and proteosomal dysfunction (Langston *et al*., 1983; Betarbet *et al*., 2002; McNaught *et al*., 2004). Initial models failed to fully reproduce all the features associated with PD, for example LBs, a pathological hallmark of the disease. However, subsequent attempts, consisting of the chronic infusion of rotenone in rodents (Betarbet *et al*., 2000) and of MPTP in mice (Fornai *et al*., 2005) have recapitulated this pathological feature of PD in addition to producing SNCA aggregates. These findings support the notion that sporadic PD results from environmental toxins that act by inhibiting the mitochondrial respiratory chain to induce selective dopaminergic cell loss.

Complex I of the mitochondrial electron transport chain (METC) consists of 26 subunits and acts as the opening gateway to the METC (Mizuno *et al*., 1989; Parker *et al*., 1989; Bindoff *et al*., 1991; Mann *et al*., 1992; Beal *et al*., 1993). It has been shown that a complex I defect can result from a depleted state of endogenous mitochondrial DNA (mtDNA), due to either a single or multiple mutations in the genes encoding for this protein complex (Swerdlow *et al*., 1996). Henceforth, the expression of mtDNA-derived defects in neuronal host cells, such as a SH-SY5Y neuroblastoma cell line, inhibits recovery from intracellular calcium transients (Sheehan *et al*., 1997), increases the production of harmful reactive oxygen species (ROS) (Swerdlow *et al*., 1996) and alters the activities of several antioxidant enzymes (Cassarino *et al*., 1997), including superoxide dismutase (SOD) (Kurtz, 2004), catalase (CAT) (Doctrow *et al*., 2003), glutathione peroxidase (GSHPx), glutathione reductase (GR) and thioredoxin/thioredoxin reductase (Arnér & Holmgren, 2000), peroxiredoxin (Ree, 2006), and glucose-6-phosphate dehydrogenase (G-6-PDH) (Tome *et al*., 2006). Although scavenging systems are in place to reduce the cellular build-up of toxic by-products (Hockenbery *et al*., 1993; Kluck *et al*., 1997), ROS exposure leaves cells more susceptible for undergoing apoptosis (Swerdlow *et al*., 1996).

The use of the pesticide rotenone is a common approach to model PD in rats (Betarbet *et al*., 2000). It crosses cellular membranes freely, independent of a DA transporter to gain access to the cytoplasm of dopaminergic neurons (Betarbet *et al*., 2000; Greenamyre *et al*., 1999). Following this, the toxin accumulates in mitochondria where it inhibits electron transportation at complex I (NADH CoQ1 reductase) of the METC (Earley *et al*., 1987), impairing oxidative phosphorylation (Schuler & Casida, 2001), and stimulating superoxide production and mitochondrial matrix dehydrogenases at proximal redox sites, leading to DA neuron apoptosis (Fiskum *et al*., 2003).

Mitochondria in differing cell types vary widely in terms of size, shape and number present. Mammalian cells typically contain between 800 to 2500 mitochondria. The numbers of mitochondria in cells differ according to the energy needs of the cell. For example, the purpose of human erythrocytes is merely the transportation of oxygen to tissue, and therefore do not contain any mitochondria whatsoever (Garrett & Grisham, 1995).

Each mitochondrion is surrounded by an outer membrane that allows for the free diffusion of large molecules into and out of the organelle, in addition to a far more complex inner membrane that is relatively impermeable and contains the electron transport enzyme complexes. Situated between the two membranes is the intermembrane space. The inner compartment of the mitochondrion is enclosed by the inner membrane to form the matrix in which the Krebs cycle takes place. During the Krebs cycle 2 electron carriers are generated, namely the reduced coenzyme (NADH) and reduced flavoproteins ($FADH_2$), which are oxidized in the inner membrane and contain a chain consisting of electron carriers. These 2 molecules function as electron donors to the series of transport enzymes that are located either inside or on the inner mitochondrial membrane (see Figure 1). Concomitantly, the ejection of protons across the inner mitochondrial membrane results in an electrochemical proton gradient, which stores potential energy.

The electron transport chain consists of five enzymatic complexes (Wallace, 1992) and is located within the inner mitochondrial membrane. The first electron carrier involved in the oxidation of most metabolites is nicotinamide adenine dinucleotide (NAD^+). The dinucleotide is formed by linking the two phosphate groups of two nucleotides. Another electron (hydrogen) carries FAD or flavin adenine dinucleotide, and derives from vitamin B_2 or riboflavin. An important feature of this molecule is that, in combination with the appropriate proteins, accept two H atoms to become $FADH_2$. FAD forms a prosphetic group, as a permanent attachment to its apoenzyme. This is in contrast to NAD^+, which moves from one dehydrogenase to another.

Four enzymes (complexes I-IV) transport electrons, ranging from NADH, succinate and oxygen. The complexes pump protons into the mitochondrial intermembrane space, thereby forming an electrochemical gradient. Complex I [NADH dehydrogenase (ubiquinone)], forms the

main entry port to the electron transport chain and is composed of 26 subunits, of which seven are encoded by mitochondrial DNA. Complex II [succinate dehydrogenase (ubiquinone)] forms another entrance to the electron transport chain, and consists of 5 subunits that are encoded by nuclear DNA. Complex III (ubiquinol-cytochrome-*c* reductase) consist of 11 subunits, with one of these encoded for by the mitochondrial DNA, and complex V (ATP synthase) which is composed of 12 subunits, with 2 subunits encoded for by the mitochondrial genome. ATP synthase uses the generated electrochemical gradient to synthesize ATP from ADP (Lee & Martens, 1986).

Although the exact relevance that rotenone holds in relation to the etiology of PD remains unknown (Perier *et al*., 2003), PD postmortem studies strongly implicate for the involvement of oxidative damage and mitochondrial impairment in the pathogenesis of the human disease (Dexter *et al*., 1989). Reports of reduced activity of complex I (NADH dehydrogenase/ubiquinone) of the METC in post-mortem PD brains (Schapira *et al*., 1990; Reichmann *et al*., 1990) support these postulations. Furthermore, some investigators have detected complex I defects in muscle-cells (Bindoff *et al*., 1991; Shoffner *et al*., 1991) and blood platelets (Parker *et al*., 1989; Benecke *et al*., 1993; Haas *et al*., 1995; Swerdlow *et al*., 1996; Gu *et al*., 1998) of PD patients. However, this issue remains controversial, with some studies failing to support previous claims of a complex I deficiency in peripheral PD cells (Mann *et al*., 1992; Reichmann *et al*., 1994; Hanagasi *et al*., 2005).

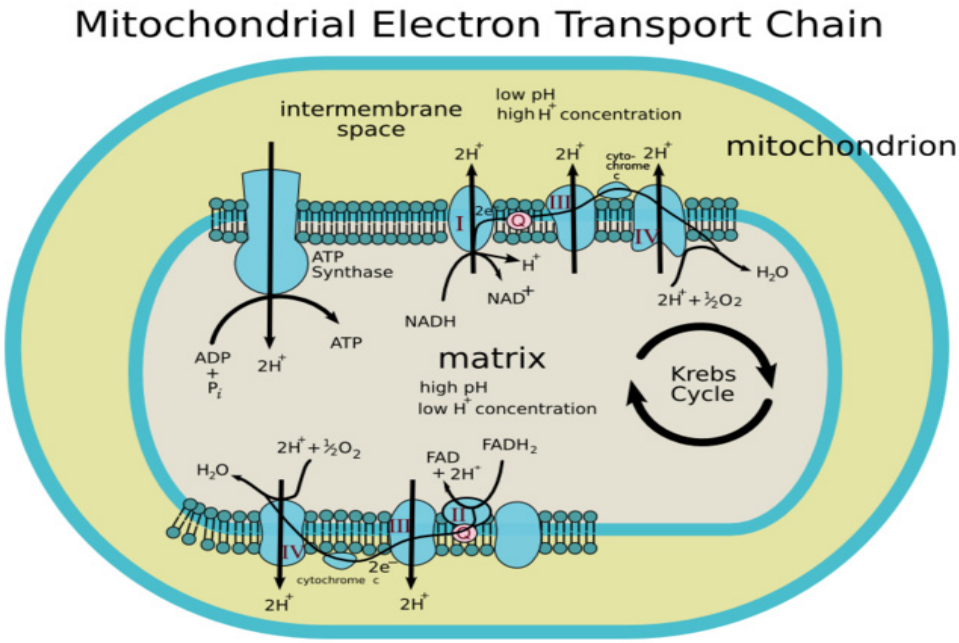

Fig. 1. A cartoon illustrating the structure and function of the mitochondrial electron transport chain. Taken from www.dialogica.com, accessed on 29/05/2008.

Microtubules seem to be an additional intracellular molecular target of rotenone (Brinkley *et al*., 1974; Marshall & Himes, 1978). Microtubular depolarization disrupts the microtubule-based transport of DA vesicles, leading to the selective degeneration of nigral DA neurons, thereby simulating PD-like symptoms in rats (Jiang *et al*., 2006; Ren *et al*., 2005). Experimental evidence for this alternative non-mitochondrial mechanism of rotenone derives from the finding that the microtubule–depolymerizing drug colchicine effectively mimics rotenone's action, while the microtubule stabilizing drug taxol, attenuates the toxin's effects (Ren *et al*., 2005).

Statins are cholesterol-lowering drugs administered to hypercholesterolemic individuals to normalize their elevated plasma low-density lipoprotein levels (LDL) (Stüve *et al*., 2003), reduce triglyceride levels and augment high-density lipoprotein (HDL) levels (Doncheva *et al*., 2006; Davidson *et al*., 2003). They currently rank among the most widely prescribed agents for preventing cardiovascular disease, decreasing cardiovascular events and for improving survival rates following both secondary and primary heart disease (Martin *et al*., 2001; Stüve *et al*., 2003). The drug acts by competitively inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a key regulatory and rate-limiting enzyme present in the biosynthetic pathway of cholesterol, thereby preventing the biological activities of L-mevalonate by catalyzing mevalonate (Ginsberg, 1998; Michal, 1999). Since L-mevalonate is an intermediate step for isoprenoid and cholesterol production, statin-therapy prevents the formation of both (Mason, 2003). Although the described events occur predominantly in the liver, resulting in, for example, an upregulation of hepatic high affinity receptor levels for LDL (Goldstein & Brown, 1984), other tissues may also partake in the process (Brown & Goldstein, 1980; Goldstein & Brown, 1990).

The brain is the most cholesterol-rich body organ, containing approximately 25% of total body levels. In order to maintain homeostasis, cholesterol synthesis, removal, storage, and transport is strictly regulated. The majority of the brain's cholesterol is synthesized locally in the CNS, since transportation via peripheral circulation prevents entering of the brain's immediate environment by an intact blood-brain barrier (BBB), except for the oxidized cholesterol products 27-hydroxycholesterol and 24S- hydroxycholesterol (Papassotiropoulos *et al*., 2000). Cholesterol is an essential component of cell membranes as well as being necessary to maintain synaptic plasticity and it is believed that astrocytes supplement the adult brain, since endogenous cholesterol synthesis is reduced in mature neurons (Poirier *et al*., 1993; Pfrieger, 2003). However, molecular and mechanistic evidence, as well as longitudinal, population-based studies suggest for a direct role played by hypercholesterolemia in the pathogenesis of numerous neurological disorders. This includes the findings that amyloid beta (Aβ) peptide

aggregates, a pathological hallmark of the AD brain, preferentially bind cholesterol (Avdulov *et al.*, 1997) and increase brain cholesterol levels during the early stages of AD progression (Kivipelto *et al*., 2001). Moreover, statins also decreased intracellular and extracellular amyloid load in cultured hippocampal and mixed cortical neurons, while an *in vivo* study demonstrated the reduced effects of simvastatin treatment on cerebral Aβ isoform levels in cerebrospinal fluid and brain homogenates (Fassbender *et al*., 2001). Mounting evidence suggests that in addition to their potent effects on plasma lipid concentrations, statins exert additional pleiotropic effects that may be independent to their primary lipid-lowering actions (Liao, 2002). These include endothelial protection *via* actions on the nitric oxide synthase system (Bellosta *et al*., 1998), antioxidant (Molcányiová *et al*., 2006), anti-inflammatory (Selley, 2005), and anti-platelet actions (Ferroni *et al*., 2006).

Six statins are currently available for clinical use. Simvastatin is a fungal-derived lipophilic statin capable of crossing the BBB (Lu *et al*., 2007). Due to its lipophylic nature, and being only 4.19 kDa in size, it readily crosses the BBB, after which it exerts effects on the CNS. Approval has been granted for its application in the treatment of patients diagnosed with diabetes mellitus and/or peripheral vascular disease (Schmeer *et al*., 2006). In addition, several studies suggest that statins may have potential therapeutic uses in strokes (Law *et al*., 2003), AD (Vega *et al*., 2003), multiple sclerosis, primary brain tumors, traumatic brain injury (Lu *et al*., 2004), as well as being responsible for an upregulation in D_1 and D_2 receptors, thereby offering potential therapeutic benefit for treating PD (Wang *et al*., 2005a; Rajanikant *et al*., 2007).

However, the inhibition of an enzyme that plays a key part in regulating cholesterol synthesis, and indirectly membrane integrity, does raise some concern. For instance synaptic function may become disturbed when cholesterol levels decrease unrestrictedly (Mauch *et al*., 2001). Adverse events associated with statin treatment have been reported, including statin-induced hepatotoxicity, dose-independent myotoxicity, as well as a number of fatal cases resulting from statin-induced rhabdomyolysis and cerebral hemorrhage (Johnson *et al*., 2005; Talbert, 2006; no authors listed, 1998; Black *et al*., 1998; Davidson, 2001; Iso *et al*., 1989).

A recent study demonstrated that short-term therapy with simvastatin induce changes in the dynamics and survival of oligodendoglial processes in cultures of rat newborn and human fetal oligodendrocyte progenitor cells as well as human adult oligodendrocytes (Miron *et al*., 2007). The human-derived cells were more sensitive relative to the rodent cells, and susceptible to increased cell death. This highlights the importance of considering the short- and long-term effects of statins, particularly the potential for inducing an immunomodulatory effect on neural cells known to be affected in multiple sclerosis. Mitochondrial impairment, as evidenced by a disturbed membrane potential, mitochondrial swelling and functional analysis of the enzymatic complexes of the METC, may proclaim a prominent place in the etiology of these serious side

effects (Amacher, 2005; Chan *et al*., 2005, Dykens *et al*., 2007). Evidence for this includes an inhibition of coenzyme Q10 (CoQ) synthesis, that plays a critical role in regulating normal mitochondrial function, when cholesterol biosynthesis was inhibited (Rundek *et al*., 2004). The supplementation of coenzyme Q10 seemingly reversed these effects (Nawarskas, 2005).

The purpose of the present study was to determine whether or not the active chemical component of simvastatin provide protection against mitochondrial damage. Male SD rats were treated with simvastatin 14 days, after which they received a single unihemispheric injection of rotenone in the substantia nigra or an equal volume of vehicle. Performance assessment in apomorphine-induced rotational asymmetry and the vibrissae-evoked forelimb placement test was performed, after which the animals were sacrificed; mitochondrial-enriched fractions isolated from the SN and the samples processed for proteomics analysis with the results compared to placebo-fed controls.

2. METHODS AND MATERIALS

2.1. Animals

Adult male SD rats, weighing between 300 and 320g at the beginning of the experiment, raised within the Animal Research Facility of the University of Stellenbosch were used for the purpose of this study. The animals were housed under standard laboratory conditions of 12 h light/dark cycles, with the temperature set at 22 ± 1 °C and humidity maintained at 60 ± 5%. All testing and training of the animals were performed during the light phase of the cycle at a correspondingly similar time of day (09:00-13:00). Food and water were provided *ad libitum*. All experimental procedures were performed in accordance with the guidelines of the Committee for Ethical Animal Research of the University of Stellenbosch.

2.2. Experimental design

The following study was designed and implemented: 48 male Sprague-Dawley rats were randomly divided into one of four groups: placebo + vehicle (P + V) (*n* = 12), placebo + rotenone (P + R) (*n* = 12), simvastatin + rotenone (S + R) (*n* = 12), or simvastatin + vehicle (S + V) (*n* = 12). Rats were treated for 14 days with simvastatin after which they received stereotaxic injections of vehicle or rotenone. Rats were tested for apomorphine-rotational asymmetry and non-drug induced motor behaviour at three separate time-points: before the placebo/drug treatment commenced in order to obtain a baseline-score, by the end of the drug treatment regime, and 5 days after stereotaxic lesioning with rotenone. A day after the final test, the animals were sacrificed, the SN dissected, and the samples snap-frozen in liquid nitrogen, for

subsequent proteomics analysis. Figure 1 provides a time-sequence of the experimental protocol.

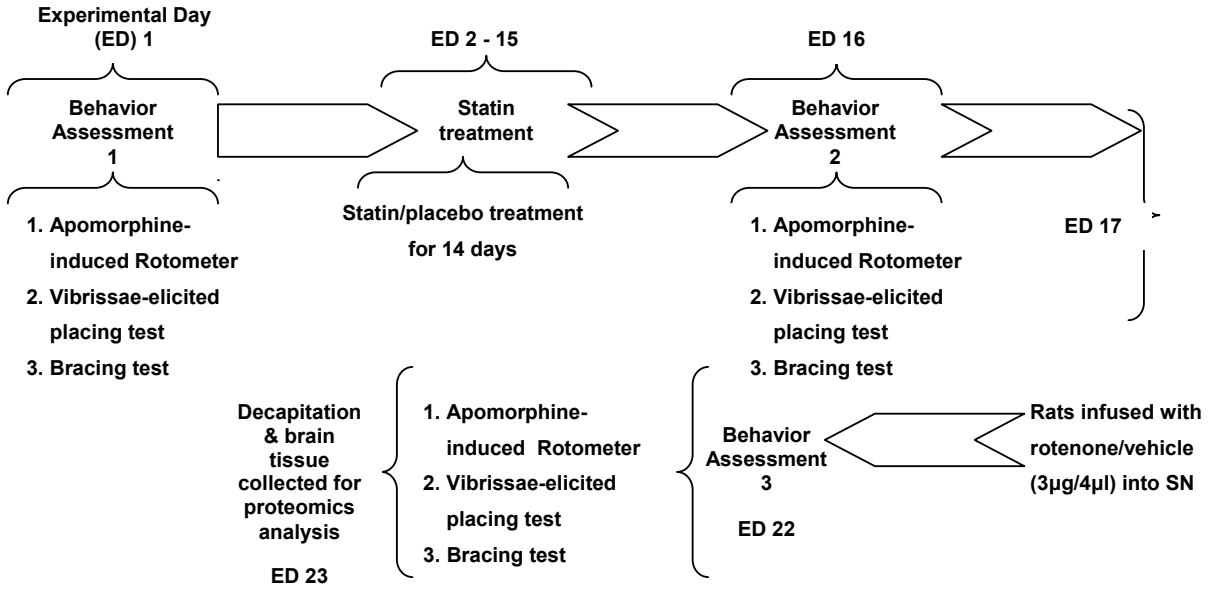

Fig. 2. A time-line by which the experimental protocol was executed. The protocol consisted of drug treatment, behavioral assessment, followed by animal sacrifice and brain tissue collection.

2.3. Drugs and chemicals used

Rotenone, apomorphine were procured from Sigma Chemicals (St. Louis, MO, USA), while the active chemical compound of simvastatin was kindly donated to us by Ranbaxy Laboratories (Dewas, India).

2.4. Drug treatment

For 2 weeks prior to the unilateral infusion of rotenone into the SNc (see below), simvastatin was administered orally on a daily basis. Two weeks of training was spent on training the rats to eat a gelatin-cube consisting of flavoured sugar. A drug-treatment regime was then followed consisting of dissolving 6 mg of the active chemical component of simvastatin in 50 µl of absolute Ethanol (EtOH) in an Eppendorf microtube, which was mixed with the gelatin-solution. For preparing a single gelatin-cube, a drop of the gelatin solution in an ice-tray using a Pasteur pipette, while 50 µl of the EtOH-dissolved statin-solution was re-suspended in the sugar solution with a sterilized pipette. After the solution had set completely, the gelatin-cube was removed from its plastic holder. A single cube was given to each rat every day for 14 days, between 13:00 and 13:30, until the day preceding surgery. During feeding, the experimenter remained

present in the room, to ensure that the rats ingested the sweet in its entirety. Control rats were treated with placebo, i.e a gelatin-cube with EtOH but without any simvastatin.

2.5. Surgical procedure

The stereotaxic coordinates for the SN were determined *a priori* by injecting Indian ink. The injection was verified histologically in an animal of the same strain, sex and weight range, to ascertain the optimal location of the tip of the injection cannula (30G needle). Reference was made to a stereotaxic atlas of the rat brain (Paxinos & Watson, 1986), and the final stereotaxic coordinates for the SNpc injection established: 5.3 mm posterior to Bregma, 2 mm lateral to the midline and 7.5 mm from the dura. These coordinates were used throughout the study for all experimental groups. For the rotenone/vehicle injections, adult male experimental animals, weighing between 375-390 g were randomly assigned to their various experimental groups before surgery. Rats were anesthetized with a combination (0.1 ml/100g, i.p.) of ketamine hydrochloride (Anaket-V®, Bayer Healthcare, South Africa) and medetomidine hydrochloride (Domitor®, Pfizer, South Africa). The surgical site was prepared by clipping the animal's head-hair and disinfecting the site with 70% EtOH. The rat was immobilized in a stereotaxic frame (David Kopf Instruments, USA) in a flat-skull position. A rubber heating-pad (REX C10, RKC, 19.0~99.9°C heat generator, Electronic Services, University of Stellenbosch) maintained the rat's body heat during surgery. A 2 cm mid-saggital skin incision was made through the scalp to expose the skull. A burr-hole was drilled at the predetermined coordinates and an infusion cannula consisting of a sterilized length of 30 gauge stainless steel hypodermic tubing was stereotaxically advanced to a depth where the internalized tip was located within the SNc cell bodies. A stock solution of the rotenone solution was freshly prepared by dissolving rotenone in the vehicle (dimethyl sulphoxide (DMSO):Polyethylene glycol (PEG), at a 1:1 ratio). Each rat received 3 µg of rotenone/4 µl of vehicle. The solution was infused through a tube attached to a Hamilton syringe over a period of 5 min using a 341-A model syringe pump (Sage Instruments, USA). Following the infusion, the needle was left in situ for an additional 5 min, before slowly retracting it from the brain. The drilled burr-hole through the skull was filled with sterilized oxidized cellulose (Ethicon, U.K.), the incision was sutured and the skin coated with an iodophor (Betadine®, Purdue Pharmaceutical Products, South Africa). To recover, the rats were placed in individual cages under heating lamps and a post-operative pain reliever (Temgesic, 0.01 mg/kg, i.p.) was administered. The condition of the sutures, the rat's food and water in-take and behaviour were monitored daily to ensure full recovery.

2.6. Motor-function assessment

Upper extremity sensorimotor function was assessed by means of tests that are sensitive to the degree of degeneration of the nigrostriatal dopaminergic projection neurons. The tests included the vibrissae-evoked forelimb placing test, which measures spontaneously induced motor asymmetries, and the apomorphine-induced rotometer test.

2.6.1. Vibrissae-evoked forelimb placing test

At 14 days post-lesion, the degree of forelimb placing asymmetry was quantified using a vibrissae-elicited forelimb placing test, which is sensitive to the sensorimotor or proprioceptive capacity (reviewed by Schallert *et al*., 2000). Briefly, the experimenter held the animals aloft by the torso in such a way that the forelimbs were allowed to dangle freely. Before commencing with each trial assessment, the experimenter moved the animal gently up and down in space, with intermittent landing of all four limbs onto the tabletop surface, thereby facilitating muscle relaxation and preventing the animal from struggling against the experimenter's mild grip. On the scoring trials, the vibrissae on each side were brushed against the edge of a tabletop, to test vibrissae-evoked forelimb placing independently on each side of the body. The forelimb not being tested was gently restrained by the experimenter by placing a finger in front of the forelimb. The sensory input to the vibrissae signals the presence of a stable surface, and the intact animal will immediately place a forelimb onto the table (Schallert *et al*., 2000; Schallert *et al*., 2002). In contrast, rats with severe striatal or nigrostriatal injury show an impaired ability to place the forelimb contralateral to the damaged hemisphere (Schallert *et al*., 1989), while still placing the unimpaired limb reliably (Schallert *et al*., 2000; see movies at www.schallertlab.org). A 4-point scale, adapted from Anstrom *et al*. (2007) was used for rating each successful, partial and non-placing response given, assessed by an assessor blind to the treatment of the animal. A score of 3 was allocated if vibrissae stimulation generated a vigorous forward and upward movement of the limb, enabling the paw to make solid contact with the table top. A score of 2 was given each time the rat placed one forelimb on the edge of the tabletop rather than the upper surface. A score of 2 was given if there was a delay in placing that exceeded 2 seconds or if there was a movement of the forelimb that did not reach the table at all. A score of 0 was given if the rat made no response with the forelimb upon vibrissae stimulation, the trial scored zero. To determine the degree of successful placing by the contralateral forelimb, these scores were totalled over the 20 trials and averaged for each animal. In addition, as a second index that is more widely used in PD models, the percentage of completely successful contralateral forelimb placing responses to vibrissae stimulation was calculated for each side by multiplying the ratio number of successful responses/total number of trials (n = 20) and multiplying by 100. Trials that received a score of 3 were considered "successful placements", while trials scoring 2,

1 and zero were considered unsuccessful attempts. Asymmetry was calculated by dividing the percentage of successful contralateral forelimb placing trials by the percentage successful ipsilateral forelimb trials and multiplying this number by ten (Anstrom *et al*., 2007). Trials during which there was any non-limb movement, detectable muscle tension, struggling, or premature forelimb movement were not scored. Each time-point assessment for this particular test consisted of 2 sessions × 10 trials from each side, with the mean score of the two test sessions considered representative. The data were analyzed using analysis of variance (ANOVA), with the critical P value set at 0.05.

2.6.2. Apomorphine-induced rotation test

Animals with severe unilateral DA depletion exhibit asymmetrical rotational behavior in a contraversive direction in response to the DA agonist apomorphine (Borlongan *et al*., 1995; Ungerstedt & Arbuthnott, 1970). The infusion of rotenone to specifically damage the dopaminergic nigrostriatal pathway, results in progressive stereotypic rotations following the administration of amphetamine or apomorphine (Alam *et al*., 2004; Heikkila *et al*., 1984). Here apomorphine-induced rotational behavior was assessed, as had been described in earlier reports (Sindhu *et al*., 2005). The degree of apomorphine-induced stereotypic asymmetry was assessed for all four groups by challenging with 0.75 mg/kg apomorphine hydrochloride (Sigma), dissolved in 0.2% ascorbate in 0.9% saline, injected subcutaneously (in the scruff of the neck). Thirty min after the injection the animal was placed in a spherical rotometer (with a diameter of 25 cm) for 60 min. The animals were video-recorded from a ventral perspective and the amount of ipsiversive (towards the lesioned hemisphere) and contraversive (away from the lesioned hemisphere) was analysed with using Noldus Ethovision software (Noldus Information Technology, The Netherlands). For each assessment, the total number of half (180°) turns in left and right directions was counted for the 60 min time interval. Analysis of data was based on net ipsilateral (right-left) turns and on net contralateral (left-right) turns. The amount of clockwise half-circle (180°) rotations (ispsilateral to the lesion) was scored as positive turns, and counterclockwise turns were counted as negative turns. The net rotational asymmetry score was calculated by subtracting the number of ispsilateral turns from clockwise turns made over the 60 min duration of the trial, with recording starting half an hour following the injection of apomorphine (Sun *et al*., 2005).

2.7. Animal sacrifice and brain tissue collection

At the end of the experiment, the rats were decapitated and the brain removed from the skull using the flat end of a spatula. The left and right SN were micropunched (see Fig. 3) and immediately frozen and stored under liquid nitrogen.

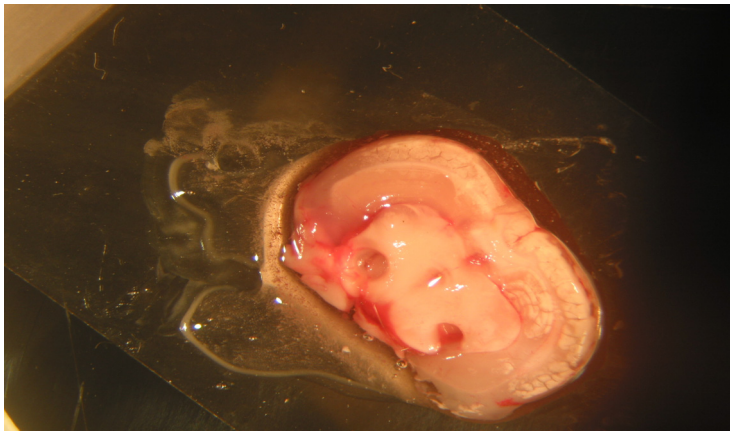

Fig. 3. A photograph to illustrate the micropunched-out SN taken from a coronal section. The SN sections for each group was pooled prior to subjecting them to mitochondrial isolation.

2.8. Isolating the mitochondria prior to proteomics

Since abundant proteins are sometimes exclusively analyzed by mass spectrometry (Aebersold & Goodlett, 2001; Haynes & Yates, 2000) sample fractionation and enrichment of lower abundance proteins are necessary to achieve a better characterization of the proteome of interest. The tissue samples, were removed from the -80°C fridge and placed on ice. Mitochondrial fractions were isolated from dissected brain tissue using a commercially available kit (MITO-ISO1, Sigma-Aldrich, USA). The tissue was weighed and then cut into smaller pieces with a scalpel-blade on a glass plate cooled with liquid nitrogen vapours. The samples were pooled and homogenized with a glass homogenizer in 10 volumes of extraction buffer. The buffer contained 10mM HEPES, 200mM Mannitol, 70mM Sucrose, 1mM EGTA and Bovine Serum Albumin to remove excess lipids, with the pH set at 7.5. The homogenate was transferred to a 2 ml Eppendorf tube and kept on ice. In order to obtain a mitochondrial fraction of ultra-high concentration, and to minimise the presence of contaminants, such as lysosomes or peroxisomes, the homogenate was centrifuged at a differential gradient. Centrifugation first occurred at a low rate of 1000 × *g* for 5 min, after which the supernatant liquid was transferred to a new tube and then centrifuged again at a higher rate of 3500 × *g* lasting for 10 min. The supernatant was removed and the pellet resuspended in 10 volumes of extraction buffer. The centrifugation steps were repeated, and the pellet resuspended in storage buffer, containing 10mM HEPES, 250 mM sucrose, 1 mM ATP, 0.08 mM ADP, 5 mM sodium succinate, 2 mM K_2HPO_4, and 1 mM DTT, pH 7.4.

2.9. Proteomics analysis of the isolated mitochondria

Conventional approaches for studying the effects of pharmacological agents on the parkinsonian brain are limited by its focus on select molecules only, thereby excluding others as well as pathways that may be relevant also. Proteomics techniques defined as the characterization of all proteins encoded by the genome, were applied to determine the mechanism by which the behavioral changes may have been accomplished (Liu *et al*., 2007). Proteomics is particularly useful as a direct measure of gene products, rather than gene transcripts that may or may not translate into proteins.

2.9.1. Protein extraction and purification

The supernatant containing mitochondria (section 2.8) was purified using a ReadyPrep Cleanup Kit (Bio-Rad Laboratories, USA), and the protein concentration of the sample quantified with a Model 550 Microplate reader and RC DC Protein Assay Kit (Bio-Rad Laboratories) using BSA as the standard. Aliquots of the final protein solution were stored at -80˚C until further use. Two-dimensional gel electrophoresis (2-D) is a protein separation technique that allows for the separation of proteins according to charge (Isoelectric focusing, IEF) in the first dimension and by molecular mass (SDS-PAGE) in the second (Mary, 1999). 2-D was subsequently performed on mitochondrial proteins derived from the SN in order to assess differential protein expression between the placebo + vehicle-infused and the statin-treated + vehicle-infused groups of animals.

2.9.2. Electrophoretic protein separation

The extracted mitochondrial proteins were separated by IEF followed by 2-D SDS-PAGE. Separation in the first dimension was performed by mixing the appropriate amount of protein sample with a rehydration buffer, containing 8 M urea, 4% (w/v) CHAPS, 65 mM DTT, 0.2% (v/v) Bio-Lytes and a trace of bromophenol blue. The sample was loaded in the IEF tray, followed by the IPG gel strip gently being placed gel side down onto the rehydration solution and overlaying the IPG strip with 3 ml of mineral oil to prevent evaporation taking place during the rehydration process. The focusing tray was placed on a PROTEAN IEF Cell (Bio-Rad Laboratories, USA) and the software program was then run. Following IEF, the strip was equilibrated for 15 min in the equilibration buffer containing DTT, followed by an additional 15 min in equilibration buffer II containing iodo-acetamide.

2.9.3. Gel staining, image acquisition and analysis

For separation in the second dimension, the gel strip (11 cm in length with an ampholyte range of 5-8, Bio-Rad Laboratories, USA) was placed on the upper edge of the 4-12% separation gel with its backside pasted tightly along the surface of the glass plate. A marker of low molecular weight protein (5 µl) was added at one end of the IPG strip. Low-Melt Agarose was used for fixing the gel strip and to remove air bubbles. The gel was run at 200V, until the bromophenol blue ran clear of the gel strip and condensed in a single line. As soon as the bromophenol blue front had traversed the gel, electrophoresis was stopped. Completed polyacrylimide 2-D gels were stained with G-Colloidal concentrated Coomassie Brilliant Blue-R (Sigma-Aldrich, USA) for visualizing the separated proteins. Gels were scanned using a GS-800 calibrated scanner (Bio-Rad, USA) and the Quantity One-4:5.2 (Basic) software program (Bio-Rad, USA) employed to create gel images. These gel images were subsequently analysed using the PDQuest (version 8) software program (Bio-Rad, USA) to identify the differentially expressed ($p < 0.05$, student's t-test) spots between control and statin-treated animals.

2.9.4. Destaining, in-gel digestion and peptide extraction

For identifying the protein profile, the spots of interest were subjected to Mass Spectrometric (MassSpect) analysis. Briefly, the gel pieces containing the proteins of interest were manually excised from the 2-D gels followed by subjection to in-gel enzymatic digestion using trypsin (Shevshenko *et al*., 1996). Using the manufacturer's protocol, the gel plugs were processed by a MassPrep Robotic Protein Handling System (MS Technologies, UK). The samples were placed in a 96-well polypropylene microtiter plate and destained twice, using 50 % acetonitrile in 50 mM ammonium bicarbonate, followed by a rinse with acetonitrile and allowed to air dry for 10 min. They were then reduced with 10 mM dithiothreitol for 30 min, followed by subjecting them to alkylation with 55 mM iodoacetamide. These steps were followed by rinsing the samples three times with acetonitrile, followed by 100 mM ammonium bicarbonate, and finally by acetonitrile. Digestion was performed by adding to each sample a 25 µl aliquot containing 6 ng/µl trypsin. The samples were then allowed to incubate for 4.5 h at 37 °C. The initial extraction of the peptides was done using 30 µl of an aqueous solution containing 2% acetonitrile and 1% formic acid. A second extraction using 15 µl of an aqueous solution containing 51% acetonitrile and 0.5% formic acid was then performed and combined with the first extraction in a cooled second 96-well plate and stored at –80^{o}C prior to analysis by mass spectrometry.

2.9.5. Peptide separation by means of in-line Liquid Chromatography and Electrospray Ionisation Mass Spectrometry (EI-MassSpect)

The partially digested peptide mixture was contained in a 96-well microtitre plate, which was transferred to a modular CapLC and autosampler system (MS Technologies, UK). Using the method described by Wilm *et al.* (1996) and Chen *et al*. (2006), the eluted peptides were then analysed further using quadripole time of flight (ESI-QUAD-TOF) mass spectrometry (MS Technologies, UK) fitted with a nano-electrosprayer having an applied capillary voltage of 3.5 kV. Prior to analysis, the instrument was calibrated against a collisionally induced decomposition (CID) spectrum of the doubly charged precursor ion of [glu^{1}]-fibrinopeptide B (GFP). Calibration was accepted when the error obtained on all subsequent acquisitions was < 20 ppm, while the sensitivity of the machine was assessed by detecting a 500 fmol injection of GFP, with a base peak signal:noise ratio of > 50:1 on the doubly charged ion. Throughout analysis, the sensitivity and calibration of the MassSpect instrument as the chromatographic resolution of the GFP peak were checked at regular intervals. For recording spectra, the MassSpect instrument was operated in data dependent acquisition (DDA) mode over the mass/charge (m/z) range of 50-2000. During the DDA analysis, both MassSpect and tandem mass spectrometry were performed on the most intense peptides as they eluted from the column.

2.9.6. Homology protein searches

Following data acquisition, all the individual uninterpreted MS/MS spectra that had been acquired for each of the precursors within a single LC run, were combined, smoothed, the background subtracted, centred, deisotoped and centroided automatically using the ProteinLynx Global Server search engine (MS Technologies, UK). The MASCOT^{TM} peptide identification search algorithm (Perkins *et al*., 1999) was used for querying the nonredundant database at the National Center for Biotechnology Information (NCBInr) with the ProteinLynx Global Server output that was generated as a single Mascot-searchable peak list file. The search parameters were defined to include up to two missed cleavage sites, a 100 ppm tolerance against the database-generated theoretical peptide and product ion masses, and a minimum of a single matched peptide. A list of the twenty highest scoring entries was produced and each suggested protein identification was either confirmed or rejected by comparing the theoretical sequence with the observed MS/MS data. The generated dataset was searched against Matrix software (http://www.matrixscience.com/cgi/), by using a rat reference database, the IPI rat version 3.34 (http://www.ebi.ac.uk/IPI/IPIrat.html, released 02/10/2007).

Furthermore, fragments were sequence-identified by performing a database search against several publicly available databases, i.e. SwissProt, www.ebi.ac.uk/swissprot; NCBI nr, www.ncbi.nlm.nih.gov; and ENSEMBL (http://www.ensembl.org/index.html). BLAST searches (www.ncbi.nlm.nih.gov/BLAST; http://www.arabidopsis.org/Blast/) were performed on the peptide fragments obtained earlier, to further analyze non-annotated expressed sequence tag (EST) hits (Altschul *et al*., 1990).

3. RESULTS

3.1. Motor-function assessment

3.1.1. Vibrissae-evoked forelimb placing test

In accordance with others (Woodlee *et al*., 2005; Anstrom *et al*., 2007), vibrissae stimulation evoked placement of the contralateral limb. When the non-lesioned side was tested, no deficit was detected in terms of placing the unaffected paw (ipsilateral to the lesion) and therefore this data was excluded from further analysis. Only performance by the affected, contralateral limb was considered. Analysis using a 2-way ANOVA indicated a significant interaction between the two dependent variables, i.e. drug treatment and infusion ($F_{(1, 44)}$=16.26, $p < 0.01$). Post hoc analysis using a Bonferroni test detected a significant difference ($p < 0.01$) between P + V (mean 59.58 ± 0.26) and P + R (P + R, mean 8.25 ± 1.6) rats, as well as between the P + V and S + R (mean 15.83 ± 0.8) groups ($p < 0.01$). While no statistically significant difference was detected between the P + V and S + V (mean 59.83 ± 0.11) groups ($p = 1$), a difference was found between the S + R and P + R rats ($p < 0.001$). Sixty denoted the perfect placing score. Statin-use improved performance in the rotenone groups, while no improvement was detected in the vehicle group (Fig. 4).

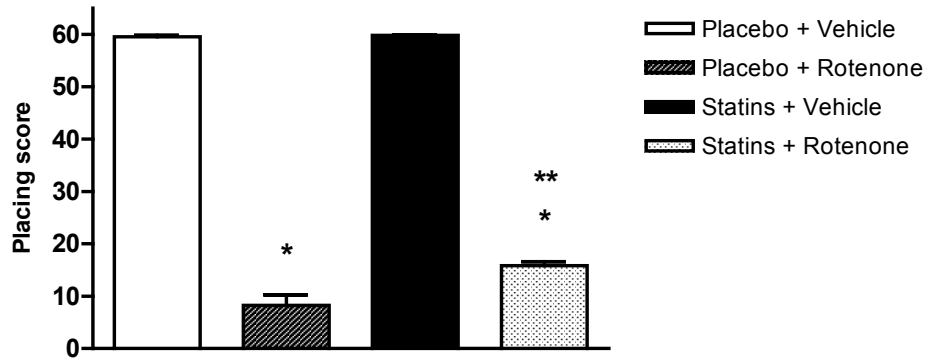

Fig. 4. Results obtained from the vibrissae-evoked forelimb placing test. The graph depicts the placing scores of the contralesional forelimb of the various groups. Rats received placebo/statin treatment for 14 days, followed by a single unihemispheric stereotaxic injection with rotenone/vehicle into the substantia nigra. Behaviour was assessed at 5 days following the intra-cerebral injeciton. Data are expressed as the mean ± S.E.M. *p < 0.001 indicates significantly different from the P + V group, while ** indicates a significant difference when comparing the P + R to the S + R group.

3.1.2. Apomorphine-induced rotation test

Rats with unilateral DA depletion show motor asymmetries characterized by a spontaneous ipsiversive bias, and the induction of a contraversive bias when treated with the DA agonist apomorphine that acts on postsynaptic D_1 and D_2 receptors, and are upregulated on the lesioned striatal side (Ungerstedt, 1971). Apomorhine's effect was obtained by analysing the summed difference between ipsilateral and contralateral half-turns (180°), providing a single independent variable, or "net rotation asymmetry score". The four groups of animals were classified according to the 2 dependent variables: statin vs. placebo, and rotenone vs. vehicle-infusion. Drug-treatment (statin vs. placebo), and infusion (vehicle vs. rotenone) were regarded as the main effects and drug-treatment*infusion as the interaction effect. The data were analyzed by means of a two-way ANOVA. This revealed significance for drug-treatment (placebo vs. statin) ($F_{(1, 44)}$= 4.5103, p = 0.04) and unihemispheric infusion (vehicle vs. rotenone) ($F_{(1, 4)}$= 206.04, p < 0.01). Subsequent *post hoc* analysis employing a Neuman-Keuls test revealed a significant difference (p = 0.04) between the rats that had received placebo only (22.67±1.96) and those treated with statins (16.79±1.96). A statistical significant interaction effect ($F_{(1, 44)}$= 4.13, p = 0.048) was detected, but a non-significant difference was obtained between statin vs. placebo-treated rats (p = 0.99), with all animals receiving a vehicle injection. For the rotenone-infused rats, significant interaction was found between statin vs. placebo-treatment (p = 0.03), implying that the significant main effect for drug-treatment resulted from the rotenone infusion, and not from the vehicle-infusion. No rotation bias was detected in the P

+ V, or in the S + V group. Fig. 5 portrays the total net number of rotations recorded for the entire 60 min duration of the trial.

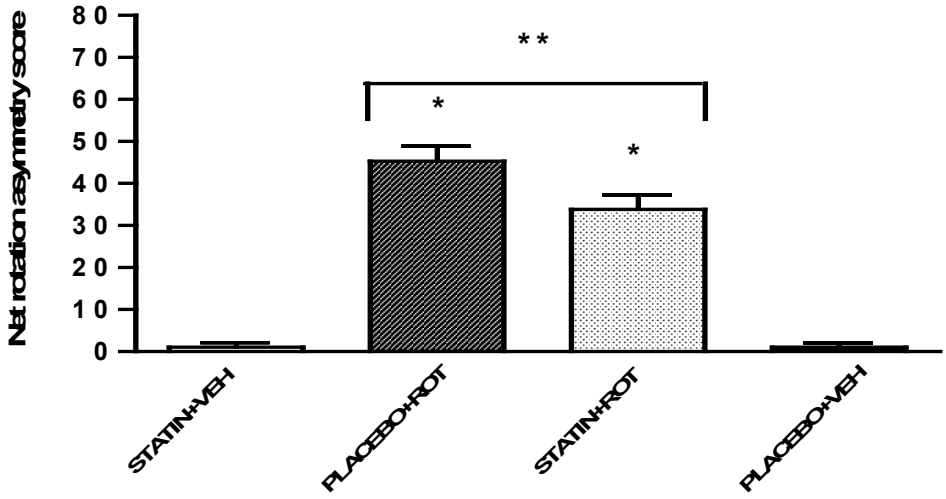

Fig. 5. Apomorphine-induced rotation response bias in rats infused with rotenone (3 µg in 4 µl DMSO:PEG or vehicle (DMSO), targeting the SNpc unilaterally. Fourteen days following surgery, the rats were subcutaneously injected with 0.75mg/kg apomorphine. All animals that received rotenone predominantly displayed ipsilateral rotations. Controls (placebo treated followed by a unilateral vehicle infusion) displayed no bias in rotation. The 'net rotation asymmetry score' was calculated by subtracting the number of half-turns (180°) made towards the contralateral (unlesioned) side from the number of half-turns towards the ipsilateral (unlesioned) side. The net ipsilateral rotations are represented as mean ± S.E.M. (n = 12). **$p \leq 0.05$ significantly different to control (P + V) animals. *$p \leq 0.05$ significantly different from statin-treated, lesioned (S + R) group.

3.2. Proteomics results

3.2.1. 2-D image analysis of SN mitochondrial proteins

Figure 6 shows the 2-D images of proteins in SN tissues of normal control and statin-treated rats, together with the molecular weights of the respective protein spots. Altogether, image analysis detected 23 protein spots, with the same parameters applying for each image.

3.2.2. ESI-QUAD-TOF Mass Spectometry identification of mitochondrial proteins

As the SN from a single animal does not yield adequate amounts of protein for reliable quantitative proteomic analysis, SN tissue from 12 rats treated with statins and 12 rats treated with placebo was pooled together. Table 1 shows the proteins that displayed significant changes in relative abundance between controls and rats treated with statins. Twenty-four proteins were found to be differentially expressed 15 of which were upregulated and 9

downregulated. In addition, Fig. 6 shows the difference in terms of spots between the P+V (Fig. 6A) and the S+V (Fig. 6B) groups. Subsequently to identification, all the proteins that showed statistical significant differences in terms of relative abundance, were classified in one of 6 broad categories describing mitochondrial-protein function (Fig 8).

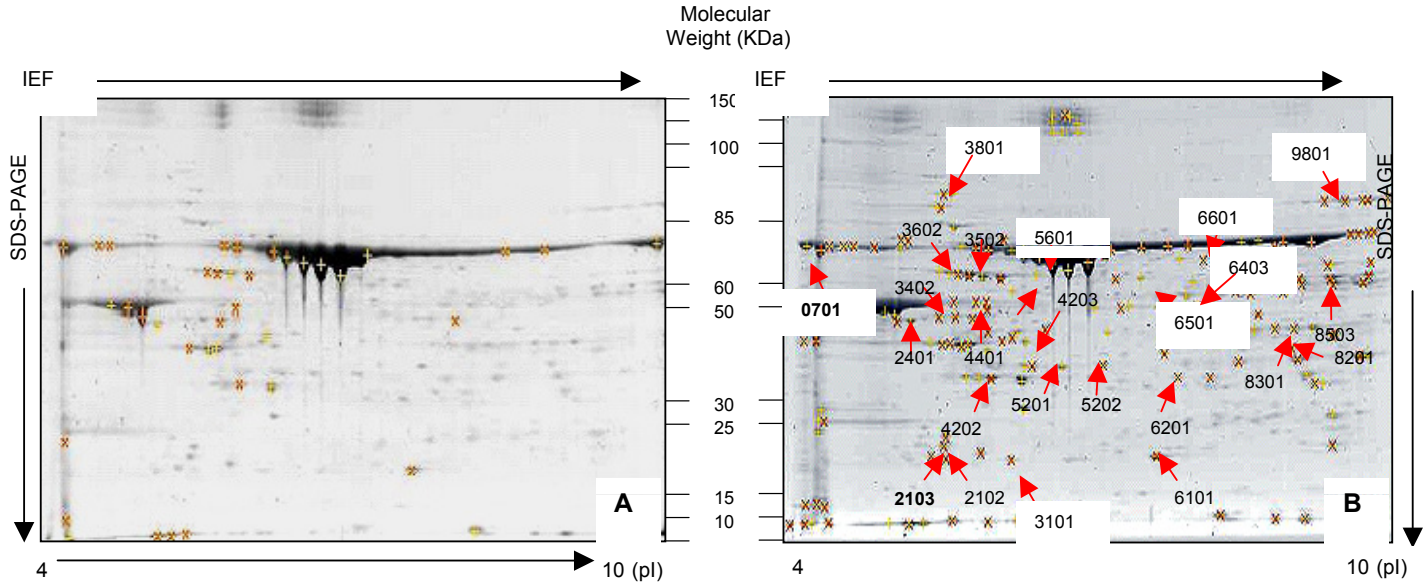

Fig. 6. 2-DE images of SN mitochondrial proteins in control (A) animals, treated with placebo for 14 days, and statin-treated rats (B), and stereotaxically infused with a vehicle. Proteomics were performed on mitochondria collected from the SN of the right lesioned hemisphere. The proteins were extracted and separated on an immobilized pH 4-10 gradient strip, followed by separation on a 12% polyacrylamide gel with SDS-PAGE and Coomassie Brilliant Blue staining. Spots found to be differently expressed to a statistically significant degree ($p < 0.05$) have been annotated. These sports were excised for the purpose of identification using Mass Spectometry.

Table 1. Relative changes in mitochondria-enriched proteins identified by Mass Spectrometry. The data represents the differentially expressed proteins when comparing placebo-controls with statin-treated animals. Only those proteins whose concentration changed to a significant extent ($p < 0.05$, student's unpaired Student's *t*-test), as determined by PDQuest and MASCOT are reported here. An SSP number was automatically assigned to each sample by the PDQuest software.

Spot no.	SSP	Mr (Da)	Protein name	Swiss-Prot accession number	Amount of peptides	Seq. coverage (%)	Fold change
1	0701	61335	Neurofilament light subunit (NF-L)	IPI00231302	4	6.6	2.91
2	2401	47141	Gamma-enolase	IPI00326412.4	5	12.7	1.12 ↓
3	2102	27378	NADH dehydrogenase (ubiquinone) flavoprotein 2	IPI00367152.1	3	12.9	1.08
4	3101	20801	Phosphatidylethanolamine-binding protein 1 (PEBP1)	IPI00230937.5	3	25.1	1.82 ↓
5	2103	24822	Ubiquitin carboxylase	IPI00204375.2	4	19.7	1.16 ↓
6	3502	60955	HSP-60	IPI00339148.2	11	21.1	1.42
7	3602	56257|	Alpha-internexin	IPI00211936.2	10	21.9	1.18
8	3402	52849	Cytochrome b-c1 complex subunit 1	IPI00471577.1	6	8.5	1.37 ↓
9	4202	38982	Pyruvate dehydrogenase E1 component subunit beta	IPI00194324.2	6	16.4	1.15
10	4203	37	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	IPI00212658.4	5	17.3	1.36
11	6101	18763	ATP synthase D chain	IPI00230838.5	2	15.5	1.18
12	3801	79412	NADH-ubiquinone oxireductase 75 kDa subunit	IPI00358033.1	4	7.0	3.07
13	4401	49490	Isoform 2 of Glial Fibrillary Acidic protein	IPI00210211.3	8	20.6	1.82 ↓
14	5201	39614	Isocitrate dehydrogenase (NAD(+))	IPI00198720.1	6	16.4	1.38
15	5202	39614	Isocitrate dehydrogenase [NAD] subunit alpha	IPI00198720.1	6	16.4	1.69 ↓
16	6403	47116	Alpha Enolase-1	IPI00768951.1	4	9.7	1.38
17	6601	73161	Dihydropyrimidinase-related protein 2	IPI00192034.2	4	6.1	1.26
18	6201	36483	Dehydrogenase antiporter	IPI00198717.8	3	9.3	1.22 ↓
19	8201	35783	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	IPI00554039.1	7	21.0	2 ↓
20	8301	43026	NAD-dependent deacetylase sirtuin-2	IPI00562798.1	3	9.0	1.47 ↓
21	8503	61416	Glutamate dehydrogenase 1	IPI00324633.2	4	7.0	4.93
22	5601	108802	PTPRF interacting protein	IPI00366315.3	1	2.4	1.3
23	9801	85433	Aconitate hydratase	IPI00421539.3	8	12.1	3.29

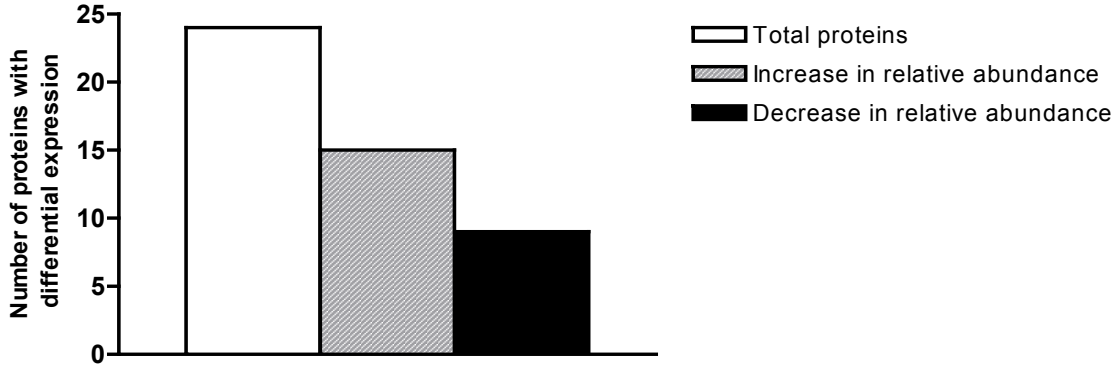

Fig. 7. Bar graphs representing the total number of proteins detected by Mass Spectrometry analysis. Those that underwent a statistically significant changeare given in white, whie the proteins that increased are represented by the grey bar, while those that decreased are shown in black.

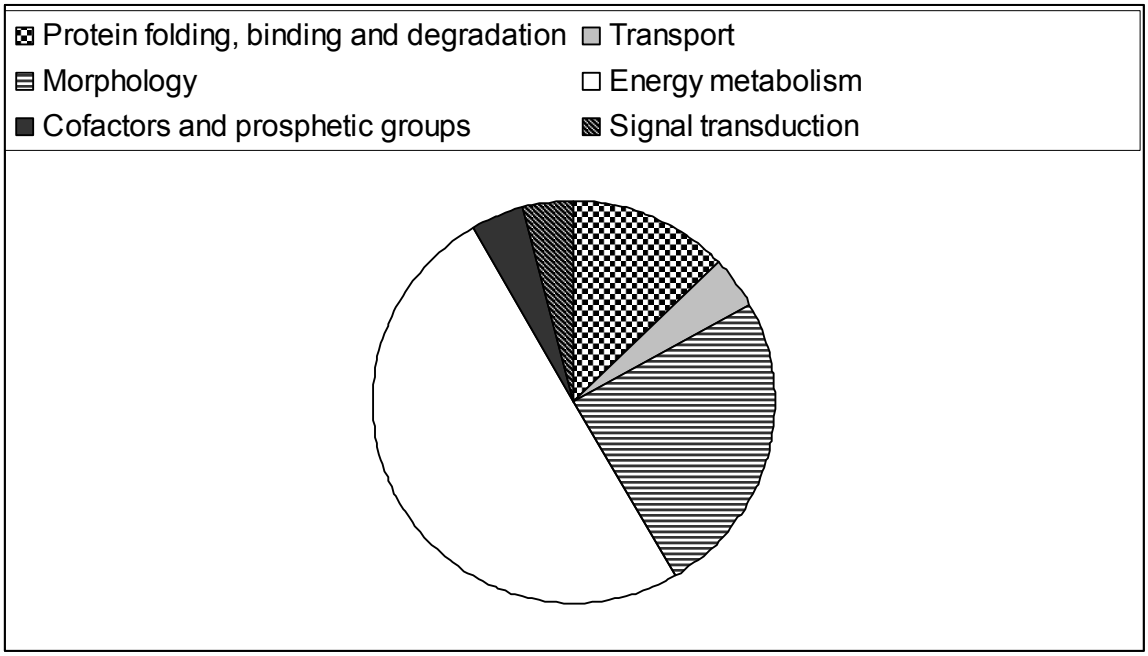

Fig. 8. The pie chart provides a functional classification of the proteins detected and represents them in proportion to each other. The identified proteins were assigned to one of six different categories, according to major functional entities of mitochondrial proteins. The figure shows that the majority (>50%) of the functionally annotated proteins are associated with energy production. Where it was possible to functionally assign a protein to more than one class, it was annotated according to its best-known function.

4. DISCUSSION

The results from both behavioral tests that were employed, the vibrissae-evoked forelimb placing test and the apomorphine-induced rotation test, show that at least a partially protective effect is exerted by the statins against the damaging effects manifesting as impaired motor function. In order to investigate what possible molecular mechanisms may underly this phenomenon, measured at the whole-animal physiology level, sensitive biochemical screens were used to assess for protein-regulated changes occurring at the subcellular level, the mitochondria. A high-concentration subcellulr fractionation consisting of mitochondria was therefore prepared, since rotenone exerts its toxic effects by targeting the mitochondrial electron transport chain, and since statins have reportedly shown selective effects on mitochondria.

For the current study, powerful proteomics techniques were applied to investigate the effects of a lipophilic statin on brain mitochondria. In addition, a behavioral profile was gathered of the

effects these drugs have in the context of the rotenone model of PD. Whereas proteomics revealed the presence of specific protein targets of statin-induced alterations in the brain, the behavioral results indicate that the drug may protect against the mitochondrial protein oxidation associate with rotenone. The proteomics results will be discussed in the context of the rat's performance in the series of behavioral tests.

A total of 23 proteins were identified by Mass Spectrometry analysis, the majority of which were expected to be mitochondria-related. Mitochondria play a fundamental role in regulating cellular physiology. In respiring mammalian cells, they are responsible for 90% of all ATP production, and contain the fundamental steps necessary for heme biosynthesis, ketone body production, and hormone synthesis (Stryer, 1988). Moreover, the oxidative phosphorylation machinery as well as the enzymes necessary for free fatty acid metabolism and those involved in the Kreb's cycle are located within the mitochondria (Mootha *et al*., 2003). Given the above, it is not surprising that when they experience mechanistic failures this may contribute significantly to a range of complex pathological conditions in humans, including diabetes, obesity, cancer, aging, neurodegeneration, and cardiomyopathy (Wallace, 1999).

4.1. Functional categorization of identified proteins

The mitochondrial genome (mtDNA) encodes for 13 polypeptides, two ribosomal RNAs (rRNAs) and 22 transfer RNAs (tRNAs) (Fernéndez-Silva *et al*., 2003). Although the exact number of mammalian mitochondrial proteins remain unknown, proteomic and genetic approaches propose the inventory of mammalian mitochondrial proteins to be at approximately 1200 (Andersson *et al*., 1998; Kumar *et al*., 2002; Lopez *et al*. 2000; Rabilloud *et al*. 1998). Of these, the functional role of 600–700 have been defined (Da Cruz *et al*., 2003; Lopez *et al*., 2000; Ozawa *et al*., 2003; Taylor *et al*., 2003; Westermann & Neupert, 2003), while the remaining ones are of unknown function.

In the present study, 23 proteins were identified that had undergone a modification in their expression levels. Most of the proteins identified through MassSpect listed in Table 1 are mitochondria related in localization and function, with a well-balanced representation of both soluble (matrix and intermembrane) and membrane-bound mitochondrial proteins. See Fig. 7 for a bar-chart display of the total number of proteins detected that had undergone significant changes, as well as the amount of these that were upregulated and those that showed significant downregulation. Moreover, Fig. 8 provides a pie chart of the functional breakdown of the 23 proteins identified. The listed proteins include components of the tricarboxylic acid cycle; proteins of the METC (complexes I–IV) and subunits of ATP synthase (complex V); components of the mitochondrial transcription and translation machinery, mitochondrial carrier proteins; chaperones (HSP-60), enzymes involved in lipid, amino acid and coenzyme metabolism, as

well as iron–sulfur cluster formation. The identified proteins will be discussed now in terms of their location in key mitochondrial pathways and the functional role each fulfill.

4.1.4. Protein folding, binding and degradation

The study found that **protein disulphide isomerase** was significantly ($p < 0.05$) downregulated (a 1.315 fold increase) compared to the placebo-treated rats. This microsomal enzyme is involved in folding newly synthesized proteins that belong to the thioredoxin family (Freedman *et al*., 1994; Gilbert, 1997) that is reduced by both thioredoxin reductase and thioredoxin. Protein disulphide isomerase was recently isolated from rat liver mitochondria (Rigobello *et al*., 2000), and its distribution in the submitochondrial compartments was localized to the outer membrane (Rigobello *et al*., 2001). The enzyme may play a critical role in the regulating mitochondrial redox functions and potentially co-operate with thioredoxin reductase in maintaining thiol homeostasis. Whereas thioredoxin reductase essentially exerts a reducing action, mitochondrial protein disulphide isomerase is thought to deliver the oxidizing counterpart of the equation. Although the major site of PDI localization is in the ER lumen, it has also been shown to localize to locate to the plasma membrane surface of a range of cell types. Observations of the presence of PDI have been made in platelets (Essex *et al*., 1995), B lymphocytes (Kroning *et al*., 1994), pancreatic cells (Akagi *et al*., 1988), hepatocytes (Terada *et al*., 1995) and thyroid cells (Couët *et al*., 1996). Although the presence of all of these are believed to be involved in transport regulation, as well as being up-regulated in response to ER stress, such as is induced by hypoxic or hypoglycemic conditions (Nomura, 2004), was suggested to contribute to the assemblage and functioning of enzyme systems endowed with a range of functions. These include pore-forming proteins, receptors devoted to protein import and to play a part in the systems that regulate mitochondrial-dependent apoptosis (Rigobello *et al*., 2001).

It has previously been suggested that the neuroprotective effects seen in diseases such as AD, multiple sclerosis, and certain variants of retinal and other eye diseases, may involve mechanisms that express heat shock proteins (HSPs) (Schmeer *et al*., 2006). The increased expression of these molecular chaperones encompass what is often referred to as the heat shock response. Recent findings suggest that unfolded or misfolded proteins participate in the neuropathology that underlie PD (Kitamura & Nomura, 2003). Of these, the expression and transcription of HSPs in the CNS are greatly modified by disease or injury (Chopp, 1993). Chaperone-like HSPs promote cell survival and suppresses apoptotic cellular events due to noxious stimuli, including heat, DNA damage and death receptor ligation (Klettner, 2004). The mechanism proposed to account for these relate to suppressing both the ‘intrinsic’ pathway, that is mitochondria-dependent, as well as to the ‘extrinsic’ apoptotic death-receptor mediated one

(Schmeer *et al*., 2006). Various neuroprotective agents show the ability to induce the heat shock response, particularly the expression of Hsp70 and Hsp72. When this mechanism is evoked in the brain, a decrease in the infarcted area due to ischemia, as well as a protection of neuronal and non-neuronal cells have been observed (Klettner & Herdegen, 2003; Klettner, 2004). This observation has spurred the hope that the induction of HSPs may provide a possible treatment strategy against various neurological disorders. For example, pretreatment with heat shock induces Hsp72 to overexpress in the striatum and offers protection against heatstroke-induced striatal injury due to the ability to reduce oxidative stress and prevent energy depletion (Wang *et al*., 2005b). Mass spectrometry analysis revealed a 1.31 increase in the degree of expression of **HSP-60**, located in the mitochondrial matrix, when compared to the placebo treated controls. This nucleus-encoded mitochondrial-resident protein (Ostermann *et al*., 1989) is a homologue of the bacterial chaperone GroEL (Cheng *et al*., 1989) and plays a critical role in mitochondrial protein assembly and folding (Okubo *et al*., 2000). Since the protective effects of HSP overexpression against several kinds of insults have been confirmed under various stress conditions (Okubo *et al*., 2000; Lau *et al*., 1997), the current findings suggest a statin-induced protection that may involve the heat shock protein chaperons.

Ubiquitin carboxylase expression levels were significantly upregulated ($p < 0.05$, 1.162) following statin treatment. This protein is believed to play a key role in degrading abnormal intracellular proteins present in eukaryotic cells (Manetto *et al*., 1988; Rechsteiner *et al*., 1987; Hershko & Ciechanover, 1986; Ciechanover *et al*., 1985). It forms part of the ubiquitin-proteasome system that is responsible for degrading abnormal, mutant or damaged proteins (Sherman & Goldberg, 2001). Empirical evidence is accumulating to suggest that when the UPS fails to degrade oxidated, nitrated or mutant proteins, LB aggregation may be provoked, with subsequent degeneration of DA neurons in the SNc (McNaught *et al*., 2001). Suppressed proteasomal activity in dopaminergic and cortical neurons results in cell death and generates proteinacous inclusion bodies that stain positive for α-synuclein and ubiquitin (Rideout *et al*., 2001; 2003; 2004; McNaught *et al*., 2002a). These preliminary findings were substantiated by PD post-mortem studies indicating reduced proteasomal activity (McNaught *et al*., 2002b) and the selective loss of the 20S proteasome α-subunit within the SNc (McNaught *et al*., 2001).The contribution of this enzyme in relation to statin-treatment should be pursued in further studies.

4.1.5. Transport

Mass spectrometry analysis identified a significant downregulation ($p < 0.05$, 1.219) of **dehydrogenase antiporter**, annotated as a mitochondrial carrier protein. Members of the mitochondrial transport family facilitate the continuous exchange of solutes by performing key steps during energy-generating pathways that exist across the inner mitochondrial membrane.

The diversity of these carrier proteins may reflect the metabolic capacity and diversity of mitochondria (Kunji, 2004). They are not only involved in the citric acid cycle and fatty acid β-oxidation (Fiermonte *et al*., 1999; Iacobazzi *et al*., 1996; Iacobazzi *et al*., 1992), but also in the synthesis and degradation of amino acids (urea cycle) and for synthesising iron–sulphur clusters and haem. Moreover, they generate heat when protons re-enter the matrix and abolish the electrochemical proton gradient (Nicholls & Locke, 1984). They also play a role in mitochondrial DNA replication, as well as in transcribing and translating mitochondrial mRNA (Cho *et al*., 1998). The proteomics results showed that mitochondrial dicarboxylate underexpressed compared to the control samples. This carrier protein is involved in exchanging malate, an intermediate of the citric acid cycle with the cytosol (Iacobazzi *et al*., 1992). It is also indirectly involved in transporting glutamate into the mitochondrial matrix, since both the aspartate/glutamate exchanger and the oxoglutarate/malate carrier form part of the malate–aspartate shuttle to remove reducing amounts of glutamate from the cytosol through a series of conversions and transport steps (Palmieri *et al*., 2001; Iacobazzi *et al*., 1992).

4.1.3. Morphology

Phosphatidylethanolamine-binding protein 1 is a basic protein that that is well represented in mitochondria. This protein displays a preferential affinity for phosphatidylethanolamine, a component of the cell membrane *in vitro* (Frayne *et al*., 2004). It widely expresses in both neuronal as well as non-neuronal tissue, including a particularly high expression found in liver and testis (Perry *et al*., 1994).. Although primarily classified as a cytosolic protein, its N- and C-terminal ends allow it to bind to cellular and model membranes, while a cavity localised to the protein surface attaches phosphatidylethanolamine with high affinity (Vallee *et al*., 2001). Additionally, this cavity site can also bind other phospholipids of a lower affinity range or opioids (Jones & Hall, 1991). When expressed in the brain, a specific N-terminal fragment of phosphatidylethanolamine-binding protein 1 known as the “hippocampal cholinergic stimulating peptide” is likely to fulfil a role in neuroendocrinology and as a transmitter (Goumon *et al*., 2004). Decreased expression levels of phosphatidylethanolamine-binding protein 1 have also been found in animal models that mimic other human neurological conditions, including AD (George *et al*., 2006) and trisomy 21 (Kazuki *et al*., 2004).

With regards to normal mitochondrial function, phosphatidylethanolamine is a specific requirement for reconstituting ubiquinone-cytochrome c oxidoreductase (complex III) of the mitochondrial electron transport chain (Nelson & Fleischer, 1981). The presence of this unsaturated lipid is critical in protecting the structure and function of cytochrome *c.* The

reduced levels observed in our statin treated group may indicate that this protective mechanism may have been compromised by statin therapy. Furthermore, a role has been assigned to phosphatidylethanolamine in α-synucleinopathies, involving the presence of missense mutations (A53T and A30P) in SNCA detected in several families diagnosed with early onset familial PD (Polymeropoulos *et al*., 1997; Kruger *et al*., 1998). Previous studies suggest that SNCA fulfils a key role in regulating brain lipid metabolism, particularly in lipid binding and their extracellular transport (Epand, 1993; Cole *et al*., 2002). A strong binding interaction between phosphatidylethanolamine and α-synuclein had been reported (Jo *et al*., 2000). If the binding of SNCA to phospholipids should be compromised, possibly due to a decreased expression of phosphatidylethanolamine, this could result in increased cytosolic concentration of SNCA, leading to fibril formation, which has been shown to have toxic effects on neurons (El Agnaf *et al*., 1998). Interestingly, the presence of one of the PD-linked point mutations (A30P) eliminated the ability of SNCA to bind to the lipid vesicles (Jensen *et al*., 1998), suggesting that SNCA's function to bind to lipids is only intact in the absence of a deranged neurological state, and may be adversely affected during PD. Although the precise functional involvement of SNCA in PD largely speculative, the overall consensus is that it fulfills a protective role (George *et al*., 1995).

Neurofilament, light polypeptide (NF-L) was found to express 2.91 times greater in the statin-treated rats compared to the placebo-treated controls. This 68kDa semi-flexible polymer forms the main structural component of axons with its principle role being to maintain axonal integrity neuronal size and shape (Lasek, 1988; Fabrizi *et al*., 2007). Recent evidence suggests that it may also serve an important function in regulating certain intracellular depositions and to protect against oxidative stress in the peripheral nervous system (Shea *et al*., 2004). It has furthermore been deemed to be an important marker for neuronal degeneration (Karlson *et al*., 1991). The protein is transported to the axons from the cell body where it has been synthesized, and is expressed specifically in large myelinated axons (Stankovic & Li, 2006). Numerous human diseases are characterized by defects in intermediate filament structure. A notable example includes recent work reporting a set of mutations in the gene that encodes for the light subunit of the neurofilament polypeptide in patients diagnosed with either the axonal (CMT1) or the demyelinating form (CMT2) of Charcot-Marie-Tooth disease, a neurological disorder characterised by a reduction in nerve conduction velocity. Animal studies support these claims, with mice null for NFL revealing viability, but displaying a 15–20% reduction in the number of myelinated axons, decreased axonal diameters and impaired nerve regeneration (Zhu *et al*., 1997). The general consensus is that underlying these conditions, a single amino acid component of the neurofilament light subunit is replaced by a mutated building block. Alternatively, sections of the N-FL gene may also be deleted or undergo duplication, thereby altering the genetic code for neurofilament light subunit synthesis. Either way, mutations of this

sort may alter the size, shape or assembly of NF-L subunits, ultimately affecting their transport towards the axonal endings. Neurofilament alterations have also been identified in PD (Trojanowski & Lee, 1998), as was suggested by recent data that neurofilament proteins, in addition to the 140-amino acid SNCA, form major constituents of Lewy bodies (Goldman *et al*., 1983). Studies that have used transgenic mice that lack the intermediate filament (IF) desmin (Milner *et al*., 2000) implicate that it plays an important role in positioning mitochondria at particular locations within the cell, within regions where the local energy requirements are relatively higher. Electron microscopic ultrastructural studies have further confirmed a mitochondria-NF interconnection revealing cross-bridges between mitochondria and NFs (Hirokawa, 1982). It was shown that the aggregation of LFs on the mitochondrial surface is mediated by the NF-H and the NF-M side-chains (Wagner *et al*., 2003). In addition, axonal mitochondria spend considerable time being immobile, undergoing elastic recoil. However, when traveling in a retrograde direction, they maintain consistent interconnections with neurofilaments (Martz *et al*., 1984; Forman *et al*., 1987; Morris & Hollenbeck, 1996; Ligon & Steward, 2000). Furthermore, it was shown that mitochondria localize and immobilize not only due to the metabolic status of mitochondria, as revealed by their ATP/ADP ratio (Overly *et al*., 1996), but Wagner *et al.* (2003) showed that mitochondrial spatial position is also directly regulated by the phosphorylation level of NFs.

Alpha-internexin (NF66) is a 66 kDa neuronal intermediate filament protein that belongs to the class IV intermediate filament proteins. It expresses primarily in the CNS axons, with embryonic neurons containing a higher proportion of α-internexin than what is found in the mature nervous system (Kaplan *et al*., 1990). Other members of this class include the neurofilament triplet proteins, namely NF-L, NF-M, and NF-H (Ching & Liem, 1991). In this study, the protein expressed significantly more than in the control brains. While it was originally named α-internexin for its ability to bind intermediate filaments (Pachter & Liem, 1985), it also expresses heavily in neurons undergoing differentiation and maturation, suggestive of a critical role played in regulating neurite outgrowth (Chiu *et al*., 1989; Kaplan *et al*., 1990; Fliegner *et al*., 1994). Moreover, it was shown to regulate the expression of other neurofilaments during neuronal development (Chien *et al*., 2005), yet α-internexin-null mice exhibited normal development (Levavasseur *et al*., 1999). Whereas it had been shown that deregulation of intermediate filament protein synthesis, defective axonal transport mechanisms, abnormal phosphorylation and proteolysis can lead to intermediate filament protein aggregation (Julien, 1999). Apoptosis-like cell death was observed following the overexpression of α-internexin in a rat adrenal pheochrompcytoma cell line with morphological changes occurring in the mitochondria, suggesting that an overaccumulation of the α-internexin gene product may play a

part in indirectly impairing mitochondrial function and subsequently inducing cell death (Chien *et al*., 2005). However, these cells belong to the peripheral NS and the relevance of these findings to the current study therefore remains to be determined.

Protein-tyrosine, receptor type, f, polypeptide interacting proteins is a member of the LAR protein-tyrosine phosphatase-transmembrane protein (liprin) superfamily that plays a critical role in a number of cellular processes. Liprins interact with members of the LAR family of transmembrane proteins, the tyrosine phosphatases that are important for axonal guidance (Burden-Gulley *et al*., 2002; Ensslen & Brady-Kalnay, 2004; Ensslen-Craig *et al*., 2005) ad is suspected of regulating the signals required to allow neurites to extend on an N-cadherin substrate (Burden-Gulley & Brady-Kalnay, 1999), and may also form complex structures and act as scaffolds for recruiting and anchoring the LAR family of tyrosine phosphatases (Schoch *et al*., 2002). In the present, study, a 1.3. fold-increase in the expression of this protein was observed.

The **dihydropyrimidinase related protein 2 (DRP-2)**, also known as collapsin response mediator protein 2 (CRMP-2) is a 64 kD protein (TOAD-64) intracellular protein involved in neuronal differentiation, axonal outgrowth and guidance (Quinn *et al*., 1999). The level of this protein was significantly increased level when statin treated animals were compared to the controls, suggesting an improved natural neuroprotective mechanism of neuronal networks as a result of statin treatment. This member of the CRMP/TOAD/Ulip/DRP family (CRMP-1-5) of cytosolic phosphoproteins, abundantly expresses in both the developing and adult brain (Veyrac *et al*., 2005). Apart from its assigned role in neurogenesis, it may also be involved in promoting microtubule formation by binding to tubulin heterodimers (Fukata *et al*., 2002). Since DRP-2 was shown to be decreased in a number of brain pathologies, including traumatic brain injury (Kobeissy *et al*., 2006), Down's syndrome (Weitzdoerfer *et al.,* 2001; Lubec *et al*., 1999) and AD patients (Castegna *et al*., 2002), it has been suggested that DRP-2 loss translates into an inability of neurons to correct impaired synaptogenesis and differentiation. However, since a significantly increased expression level for DRP-2 has also been reported to be consequential to normal ageing (Poon *et al*., 2006a), the possibility that neuronal sprouting is a regulated compensatory response to aged-induced neuronal dysfunction, should not be eliminated. The extent of involvement of this protein in neurogenic and plastic mechanisms, as was indicated by the current results, should be explored in future studies.

Glial fibrillary acidic protein (GFAP), a 50 kDa protein that forms the major constituent of glial filaments makes up the cytoskeleton of mature astrocytes (Eng *et al*., 2000). The protein is thought to be important in modulating astrocyte motility and shape by providing structural stability to astrocytic processes. Mice lacking GFAP are hypersensitive to cervical spinal cord injury caused by sudden head acceleration. This finding has provided direct evidence for

GFAP's structural role (Nawashiro *et al*., 1998). An increase in GFAP synthesis has been associated with astrogliosis, with immunohistochemical studies revealing a rapid over-synthesis of GFAP content in astroglia in the brains of AD patients (Mancardi *et al*., 1983). In addition, it has been proposed as a sensitive and early biomarker of neurotoxicity, since increased GFAP mRNA levels were detected in the substantia nigra of 6-hydroxydopamine lesioned rats (Rodrigues *et al*., 2004). GFAP mutations associate with Alexander disease, a rare neurodegenerative disorder characterized by white-matter degeneration, and the formation of cytoplasmic inclusions called Rosenthal fibers detected in the astrocytes (Alexander, 1949). Mitochondrial abnormalities have been implicated as a contributing cause for the disease (Gingold *et al*., 1999; Schuelke *et al*., 1999; Nobuhara *et al*., 2004). In the current study, GFAP was downregulated 1.818-fold compared to the control animals, possibly indicating an activation of protective mechanisms due to statin-treatment.

4.1.4. Energy metabolism

Gamma-enolase, with its γγ isoform known as neuronal-specific enolase (NSE), is a neuron-specific subunit of the glycolytic enzyme enolase. Significant difference in its relative abundance was shown being down-regulated as a result of statin-treatment. It has previously been suggested that an up-regulation in the expression of NSE marks a mobilization of cellular defence mechanisms in the damaged striatum (Valastro *et al*., 2007), with increased NSE levels detected in the CSF of patients suffering from Kreitzfeld-Jakob disease and victims of cerebrovascular accidents and cerebral trauma (Dauberschmidt *et al*., 1983; Hay *et al*., 1984; Wakayama *et al*., 1987; Jimi *et al*., 1992). In line with the neuroprotective and neurorestorative functions, as well as its ability to promote neuronal survival assigned to NSE (Hattori *et al*., 1995), an increased expression was found in the striata of $_{L}$-Dopa-induced dyskinetic rats compared to non-functionally impaired ones (Valastro *et al*., 2007).

The variation in **ATP synthase D chain** between control and PD-like animals reported here (this protein was significantly upregulated at 3.07-fold compared to the control rats) is consistent with others who reported similar results derived from human PD patients (Basso *et al*., 2004).

The expression levels of 9 proteins were reduced, including **α-enolase (ENO1)**, a glycolytic enzymatic protein, that has an evident N-terminal extension and exhibits characteristics of a typical mitochondrial targeting peptide. α-enolase is proposedly affected in age-related neurodegenerative diseases, including in PD and Alzheimer disease cases (Poon *et al*., 2006b).

Glutamate dehydrogenase (GDH) is an important branch-point enzyme localised to the mitochondria that plays an important role in carbon and nitrogen metabolism (Stillman *et al*., 1993). The current study found this protein significantly upregulated (4.93 fold), compared to the controls.

Aconitate hydratase (aconitase) was found to be significantly upregulated ($p < 0.05$, 3.29 fold) as a result of statin therapy. The enzyme is located in two subcellular compartments, cytosolic aconitase and aconitase that is mitochondrial-matrix bound, and has been implicated in the Krebs cycle (Pickworth Glusker, 1971). The majority of eukaryotic cells contain two types of aconitase. This enzyme participates in the citric acid, and is responsible for reversibly hydrating the labile, enzyme-bound intermediate cis-aconitate yielding citrate and isocitrate. In the current study, **isocitrate dehydrogenase** (NAD(+))was also found to be significantly upregulated in response to statin-treatment.

In this study, a 3.07 fold increase in the expression of **NADH:ubiquinone oxireductase** was found, a 75 kDA subunit of the inner mitochondrial membrane (Galante & Hatefi, 1979). This protein complex plays a critical role in regulating energy metabolism, by catalyzing the transport of electrons from NADH to ubiquinone for ATP synthesis, in addition to proton translocation from the mitochondrial matrix to the intermembrane space (Smeitink *et al*., 1998; Hatefi, 1985). Several reports suggest that mitochondrial enzyme deficiencies can contribute to the progressive pathology of several neurodegenerative diseases, including PD where a significant and selective reduction of complex I activity has been identified in key brain regions associated with PD (Schapira *et al*., 1990; Mizuno *et al*., 1989). Other studies also report a strong correlation between decreased complex I activity and reduced levels of mitochondrial ubiquinone (CoQ_{10}) in the PD brain (Shults *et al*., 1997; Gotz *et al*., 2000).

The current study revealed **glyceraldehyde-3-phosphate dehydrogenase**, an essential glycolytic enzyme for catalyzing reversible oxidative phosphorylation of glyceraldehyde-3-phosphate, to be upregulated (2 fold) in response to statin treatment. This important energy-yielding step in carbohydrate metabolism, depends on the presence of inorganic phosphate and nicotinamide adenine dinucleotide (NAD). Recent evidence suggests that it may be multifunctional, independent of its role in carbohydrate metabolism. Possible activities include phosphorylating transverse-tubule proteins (Kawamoto *et al*., 1986), stimulating RNA transcription (Morgenegg *et al*., 1986), interacting with microtubules (Huitorel & Pantaloni, 1985), influencing RNA catalysis by binding to hammerhead ribozyme (Sioud & Jesperson, 1996) and acting as a diadenosine tetraphosphate binding protein to influence DNA replication and DNA repair (Baxi & Vishwanatha, 1995). This protein also acts as a ‘markers protein’ that is particularly vulnerable to damage caused by excessive ROS production (Cabiscol *et al*., 2000). A similarly vulnerable one is **pyruvate dehydrogenase**, which the current study also found to be significantly upregulated at the protein level, suggesting decreased ROS production.

A slightly significant expression is reported here for **Sirt2**, an nicotinamide adenine dinucleotide (NAD)-dependent deacetylase belonging to the sirtuin family that deacetylates the 'Lys-40' of alpha-tubulin as well as histones *in vitro* (North *et al*., 2003). It shows involvement in

controlling mitotic exit during the cell cycle, probably due to its role in cytoskeleton regulation (Inoue *et al*., 2007). Sirtuins have been implicated in a broad range of biological processes, including stress resistance and glucose homeostasis, while sirtuin deacetylase activity also associates with pathways that oppose age-associated diseases, such as type II diabetes, obesity, and neurodegenerative disorders (Haigis & Guarente, 2006;). In addition, Sir2 mediates lifespan extension through a caloric restriction pathway, thereby extending lifespan in species ranging from yeast to flies (Lin *et al*., 2000; Rogina & Helfand, 2004) while its overexpression was also shown to lower cellular ROS levels (Wang *et al*., 2007).

The mitochondrial precursor **cytochrome b-c1 complex subunit 1** expressed 1.369 times less than in the control rats. This enzyme, of the cytochrome (cyt) bc_1 mitochondrial complex family form a component of complex I of the METC, catalyzing the transfer of electrons from a quinol in the lipid phase (ubihydroquinone in the classical bc_1 complexes) to a higher-potential acceptor protein in the aqueous phase. The complex plays a central part in cellular energy metabolism and has been implicated in a diverse range of mitochondrial myopathies located to mutations in genes encoding for the proteins of the bc_1 complex (Fisher & Meunier, 2001; 2002; Rose, 1998; Vogel, 2001). Although a decreased expression was found for this complex in the statin-treated rats, no compromise in motor-function was observed, and therefore any adverse effect that the drug might have had at this level did not impinge on physiological function. Although the results caution that statins might be implicated in complex I related pathology, this matter deserves further attention before a risk for this can be confirmed.

MassSpect identified the mitochondrial, tetrameric enzyme **isocitrate dehydrogenase** as expressing 1.69-fold less in the statin-treated than in the control animals. In particular, where this enzyme consists of three subunit types (Ramachandran & Colman, 1980), the alpha subunit was identified in the current experiment. As a group of enzymes, they are responsible for catalyzing the conversion of *threo*-D-isocitrate to α-ketoglutarate in the presence of the mitochondrial enzyme NAD (Haselback & McAlister-Henn, 1993). This enzyme catalyzes the oxidative decarboxylation reaction that consists of converting isocitrate to α-ketoglutarate and CO_2, by reducing NAD to NADH. In turn, NADH forms the energy storage compound ATP (Bzymek & Colman, 2007). The downturn in its expression could possibly be explained by the fact that the enzyme is under strict cellular control, being activated by ADP and inhibited by the ultimate products of the reaction, namely NADH and ATP. Therefore, when the cell does not require additional energy, the enzyme is inhibited by ATP thereby decreasing the rate of oxidation of isocitrate. On the other hand, when the cell requires energy, the conversion of isocitrate to α-ketoglutarate is promoted through the activation of ADP.

4.1.5. Cofactors and prosphetic groups

As the largest of the respiratory complexes, NADH dehydrogenase, also known as ubiquinone acts as the input site to the mitochondrial ETC. NADH dehydrogenase uses flavin adenine dinucleotide (FAD) as the redox prosthetic group, from where electrons are passed from various dehydrogenation reactions to ubiquinone, that constitute part of the “classical” respiratory chain complexes. Thus, functionally speaking, it is extremely similar to complex I of the respiratory chain. The transferal takes place via two flavoproteins, the electron transfer **flavoprotein** (ETF) and ETF:ubiquinone oxidoreductase (ETF:QO). Ubiquinone oxidoreductase (ETF:QO) is a monomer located in the inner mitochondrial membrane after being imported from the cytosol, containing a 4Fe4S cluster as well as FAD (Simkovic *et al*., 2002). Flavin binding only occurs once the enzyme is inside the mitochondrial space, forming an NADH dehydrogenase (ubiquinone) flavoprotein (Nagao & Tanaka, 1992; Saijo & Tanaka, 1995; Brizio *et al*., 2002). This constitutes an essential step for catalysing flavoprotein activity, regulating their folding, assemblage and/or stability (Nagao & Tanaka, 1992; Saijo & Tanaka, 1995; Sato *et al*., 1997; Muralidhara *et al*., 2006) and to link the oxidation of fatty acids and certain amino acids to the mitochondrial ETC (Zhang *et al*., 2006). MassSpect analysis revealed that the expression of this enzyme was significantly upregulated (1.08) compared to the control samples. An EFT dehydrogenase deficiency has been linked to the human genetic disease multiple acyl-CoA dehydrogenase deficiency (Vianey-Liaud *et al*., 1987), while multiple acyl-CoA dehydrogenation deficiency (MADD), a disorder thought to be due to inborn errors of metabolism of fatty acids, amino acids and choline may result from defects present in 2 flavoproteins. Mutations have been detected in the *ETFDH* gene of patients, coding for ETF:QO (Olsen *et al*., 2003), as well as in the electron transfer flavoprotein (ETF). The disease is marked by impaired multiple dehydrogenation reactions due to defective electron transfer from several primary flavoprotein dehydrogenase to mitochondrial respiratory chain. MADD patients were found to be responsive to riboflavin dietary supplement. Although the molecular defect in this particular form remains unknown (Gregersen *et al*., 1982), a recent study suggests that riboflavin-responsive MADD may associate with defects in the *ETFDH* gene also (Olsen *et al*., 2007).

4.1.6. Signal transduction

The **guanine nucleotide-binding proteins** (G proteins) serve as modulators or transducers in a variety of transmembrane signaling systems, by transferring stimulatory or inhibitory signals to intracellular protein targets in response to activation by specific cell-surface receptors. The G protein family is present in all eukaryotic cells, where they control a wide range of metabolic, humoral, neural and developmental functions, by coupling hormones and neurotransmitters to their cellular response targets (Stryer & Bourne, 1986; Bourne, 1986; Gilman, 1984). G protein-linked receptors (GPLR) is activated by a diverse groups of effector units. These range from the

visual G protein-linked receptor rhodopsin that detects a small numbers of photons, and are therefore involved in regulating retinal cyclic GMP phosphodiesterase (Ando *et al*., 1991; Chabre *et al*., 1993), to those found in the olfactory system for transducing olfaction-related signals from odorant receptors (Buck & Axel, 1991; Chess *et al*., 1992), as well as the operation of gustatory signals via GPLRs (Margolskee, 1993). Receptor-G protein forms a complex with the integral membrane glycoprotein adenylyl cyclase (AC), to play a fundamental role in regulating numerous physiological processes including hormone effects, metabolism, learning, and memory. In addition, a multitude of human diseases may stem from inherited or sporadic mutations of the genes encoding this component's various signaling components (Raymond, 1994). Moreover, several G protein–linked neurotransmitters inhibit N and P/Q type channels, which are voltage-gated Ca^{2+}-selective channels (Hille, 1994), thereby initiating a signal transduction cascade where the channels open less readily upon depolarization (Bean, 1989). G-proteins are generally composed of 3 units, alpha, beta and gamma, with the beta and gamma chains that are required for GTPase activity, for replacement of GDP by GTP, and for G protein-effector interaction. Specific transient interactions between these components generate the pathways that modulate cellular responses to complex chemical signals. For example, G ß γ subunits activate phospholipase A_2 (PLA_2) (Jelsema & Axelrod, 1987), while both G ß γ (Camps *et al*., 1992; Boyer *et al*., 1999) and G_α (reviewed by Sternweiss, 1994) are potential activators of the PLCβ isozymes *in vitro* and *in vivo*. The MassSpect results identified a significant upregulation (1.36 fold) of a ß subunit of G proteins that is encoded for by *Guanine nucleotide binding factor 2* (*GNB2*). Protein detection methods specifically identified the 35,000 M_rs individual variant, the other being of 36,000 M_rs (Sternweis *et al*., 1981; Sternweis & Robishaw, 1984; Neer *et al*., 1984; Evans *et al*., 1987). Although the Gßγ subunit is made up of two polypeptides, namely Gß and Gγ, Gß is functionally regarded as a monomer since the two subunits cannot dissociate except by means of denaturants (Clapham & Neer, 1997). The G beta gamma subunit was only recently recognized as a signal transduction molecule in its own right, with the first evidence that G ß γ could regulate effectors itself when it was shown that the G ßγ subunit activates a K^+-selective ion channel (I_{KACh}) in cardiac atrial cells (Logothetis *et al*., 1987). It is therefore implied that statins induced an upregulation of intra- and intercellular communication systems, possibly operating via G-protein coupled receptors.

5. CONCLUSION

A robust quantitative proteomic approach was used that identified >300 proteins from mitochondria-enriched fractions isolated from rat SN. Of these proteins, 24 showed statistical significant differences ($p < 0.05$) in terms of relative abundance expression between rats treated with statins and those on placebo only.

The data is consistent with the hypothesis that mitochondrial dysfunction (as was experimentally modeled by the central infusion of rotenone) plays a key role in the pathogenesis of PD and that treatments targeted at mitochondria might decrease the functional decline seen in PD. Although rotenone's relevance to the etiology of PD is unknown, the published data raise several important implications pertaining to its significance and reliability as a tool for inducing models of PD. Increasing evidence also suggest for the involvement of mitochondrial abnormalities in the etiology of neurodegenerative diseases other than PD, such as a complex I deficiency in the METC in the cerebral cortex and in the platelets of AD patients (Jimenez-Jimenez *et al*., 1998).

No significant adverse effects on the CNS have been reported following long-term use of statins in humans (Pedersen *et al*., 1996), while caloric restriction has been shown to increase life span and heighten resistance to various age-related disorder in rodents (review in Koubova & Guarente, 2003). Taken together, the findings from the current study provide clues for future studies to further investigate the molecular basis of the protective effects of statin-treatment on an underlying PD pathology. A better understanding of these may assist in developing therapeutic strategies for effectively treating this human neurological disease. However, it is acknowledged that the differential expression of mitochondrial proteins demonstrated in the current study, may be due to another factor, unrelated to statin treatment, remaining to be identified. The results further emphasize the power and importance that direct MassSpect-based analysis of mitochondria hold towards revealing drug-induced changes that occur at the proteome level.

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CHAPTER 8

CONCLUDING REMARKS AND RECOMMENDATIONS FOR FUTURE RESEARCH

The overall purpose of my project was to develop and ascertain 2 animal models known for mimicking the core pathology seen in clinical PD. It is becoming increasingly clear that complex diseases stem from gene-environment interactions. A new field in health research, *the developmental basis of health and disease*, emphasizes the important role that the timing of exposure to adverse pathogens could play in developing diseases later in life. The fundamental tenet for this assumption stems from a multitude of animal studies that have demonstrated that the *in utero*/developmental period is a particularly sensitive window for environmental exposures, nutritional or chemical.

As an example of an environmental influence that could worsen the pathogenesis of the disease, the study described in chapter 5 demonstrated how exposure to a particular adverse factor, maternal separation (MS) that acts as a model of early adverse experiences can induce greater neuropathological harm and motor-related deterioration in an animal model of PD, compared to non-separated control animals. What remains to be established in future studies is whether these toxic-induced pathogenic responses result from altered gene expression or altered protein regulation associated with altered cell differentiation that are involved in the interactions between cell types and the establishment of cell lineages. In addition, a thorough investigation of the type and extent of the altered morphological and/or functional character of the tissues, organs and systems should be pursued in subsequent work. These states of altered potential or compromised function that result from changes in gene expression, may be due, at least in part, to altered imprinting, and the underlining methylation-related protein-DNA inter-relationship associated with chromatin remodeling. These effects may occur in a time-specific (i.e. vulnerable window) and/or tissue-specific manner and such alterations may be irreversible. Therefore, a detailed analysis of the steps that led to establishing the end-result of the current work, namely an animal sensitized to the extent that it is more susceptible to diseases later in life, should be pursued. Moreover, future research should also attempt to tease apart the relative contribution that several factors, including genetic predisposition, stressor intensity, cognitive appraisal mechanisms and coping processes may offer towards influencing the likelihood of an individual developing a neuropathological disease. This is important since it will not only shed light on why the vast majority of individuals exposed to stressful life events do not develop a neurodegenerative disease, but also promises to elucidate on how interactive patho-mechanisms work to play a role in inducing the onset and development of neurodegenerative disease, where exposure to stressful events played a conceivable part.

The thesis also investigated claims that a commonly prescribed hypolipodemic drugs, marketed under the tradename simvastatin, may result in mitochondrial-related damage. Since this patho-mechanism is also implicated as an underlying cause of PD, significant clinical implications could be induced in patients vulnerable to mitochondrial-related disease, as PD patients are assumed to be. On the other hand, a group of pharmacological agents known as statins have been hailed for their possible therapeutic benefit to alleviate the symptomatic burden of patients suffering from a range of neurodegenerative conditions other than PD. In the case of PD, the use of statins by PD patients have been reported to increase DA output by the dopaminergic cells, providing additional motivation to investigate the role of the drug in the context of PD. Although Levodopa has been successfully applied as a valuable therapy with which to treat the disease, its application is limited as a form of long-lasting therapy by the onset of debilitating complications following long-term use. In addition, it fails to address the root of the disease, and contributes much towards an oversimplified and misleading conception of PD as a malady of the substantia nigra only, since synthetic dopamine (Levodopa) aims to replace the lost dopamine. Traditional animal paradigms that focuses essentially on the demise of nigral dopaminergic neurons, seem to fail regarding the provision of a simplistic, single mechanism view of the pathogenesis of PD, while it is commonly acknowledged that it has a multi-factorial origin, that includes mitochondrial activity dysfunction. My results show that statins provide a degree of protection against rotenone-induced neuronal insults. Since the neuropathological features evoked by this neurotoxin at a molecular and behavioral level are similar to that of the human disease, it is conceivable that statin-use may be of benefit to PD patients also. These claims deserve further investigation, particularly with regards to elucidating the functional role played by the proteins that display expressional changes, and whether their function relates to neuroprotective properties.

In both the studies reported in chapter 5 and 7, the variable factor (whether environmental or drug-induced) preceded exposure to the known parkinsonian-agent. The choice if neurotoxic agent was carefully chosen in the context of the disease mechanism that was to be investigated. In this regard, 6-OHDA was infused following subjection to an MS paradigm, for its ability to create consistent dopaminergic cell death. On the other hand, rotenone was the agent of choice to deliver to rats that had previously been treated with statins, since it is well established that it works to induce cyto-functional impairments by inhibiting complex I and III of the mitochondrial ETC.

Nerve growth factor (NGF) was seen to be upregulated in female rats compared to the males in a 6-OHDA model of PD. This finding deserves further investigation using complimentary techniques, particularly a genetic approach to ascertain whether the effect can be repeated using an NGF knock-down or knock-out paradigm. In addition, future work should

investigate whether the upregulation of NGF was modified in any way, by even the trace amounts of female gonodal steroids. Using a surgical or chemical approach to remove the gonads, or by knocking down estrogen receptors, an undisputed position on this should be attained. Should the mechanism underlying NGF's upregulation that was responsible in this model become known, whether the growth factor functions independently or in synchrony with any of the female steroidal factors, the validity and reliability of the protein to produce similar effects should be assessed in other (i.e. primate) models of PD, for possible future clinical application.

Every effort had been made to overcome the limitations that may have been imposed by resource restrictions, or other reasons. For chapter 4, the use of a lower number of rats, may have prevented certain measures from showing a greater (and statistically significant) effect. This particularly pertains to the results obtained from subjecting the tissue to HPLC-EC analysis. Furthermore, for chapter 7, It is acknowledged that the pathways induced by simvastatin in the rat model, to affect the SN's mitochondrial proteome in the way demonstrated, may differ significanty from those of humans. Future work should aim to establish the extent to which the rat and human data overlap in this regard. Moreover, the mitochondrial fractions generated from the SN of the statin and placebo-treated rats for proteomic analysis had to be pooled, in order to generate enough proteins for detection. This prevented the analysis of the changes in mitochondrial protein-levels brought on by simvastatin-use in individual rats. In addition, confirmation of the presence of these proteins detected through proteomics, were hampered by the limited availability of monoclonal antibodies generated against these proteins.

APPENDIX A

GENERAL METHODOLOGY AND MATERIALS USED

1. Animal maintenance

Sprague-Dawley rats were used for each experiment described in this Thesis. Animals were subjected to a 12-hour light/dark cycle (lights came on at 6am till 6pm). The animals were kept in clear 40 x 25 x 20cm Plexiglas cages, in an AAALAC accredited facility, in rooms with temperature 22±1°C and a 12:12 hr. light:dark cycle (lights on from 07h00 to 19h00). The rats had *ad libitum* access to pelleted rat chow and tap water. All experimental procedures followed in this study had been approved by the Committee for Experimental Animal Research of the University of Stellenbosch (Project number: N05/04/004), and were in accordance with the National Institutes of Health Guide for care and use of laboratory animals.

2. Maternal separation paradigm

MS has been described as a potent naturalistic stressor (Stanton *et al*., 1988) and has been used extensively as a model of early life stress by various investigators, i.e. Daniels *et al*. (2004), Faure *et al*., (2006), Ladd *et al*., (1996), Lubach *et al*., (1995), van Oers *et al*., (1998), Lyons *et al*. (1999), and Sánchez *et al*. (2001). Adult male-female pairs were housed under normal conditions to allow for mating. Immediately after the rat pups were born, the adult males were removed while the mothers remained with the pups. On postnatal day (PND) 1 (day zero was regarded as date of birth), rats were sexed and culled to 8 pups per dam. If there were fewer than 8 males, then females were included to make up the 8 so as to ensure equal nurturing. Litters were randomly assigned to either a control (non-separated) or MS group (n = 8/group). Beginning on PND 2, MS involved physically removing the dam from the home cage that contained the pups, for 3 hrs per day from 10:00 to 13:00, leaving the pups undisturbed in their home cage. During the separation period that occurred daily for 13 days, the pups were housed in a room away from the mother to prevent communication *via* ultrasound vocalizations from taking place (Hofer, 1994). The temperature of the room to which the rat pups were transferred was maintained between 31˚C and 33˚C with two heaters on either side of cages, recording thermometer at same location as cages, thereby minimizing the possible influence of hypothermia. After PND 14, the pups returned to normal housing conditions. Control animals were handled identically to experimental ones, except that the pups were not separated from the dam during the first 14 days after they were born. On PND 21 both groups were weaned by permanently removing them from the dam.

3. Drug treatment

For 2 weeks prior to unilateral infusion of rotenone into the SNc (see below), simvastatin was delivered daily. After 2 weeks of training the animals to eat a jellie-cube given to each rat individually, a drug-treatment regime was prepared and executed for each animal in the

following manner: 6 mg of the active chemical component of simvastatin was administered orally on a daily basis for 14 days, between 13:00 and 13:30, until the day preceding surgery. 6 mg of the drug was dissolved in 50 µl of absolute Ethanol (EtOH) in an Eppendorf microtube. A sugar solution, consisting of commercially-available jelly and gelatine, was dissolved in boiling water. For each animal a droplet of the solution was placed with a Pasteur pipette in a single container of an ice-maker, and 50 µl of the dissolved statin-solution was re-suspended in the sugar solution with a sterilized pipette. When the solution had completely stalled, the jelly-block was removed from its container, and given to each rat. Control rats treated with placebos, received a jelly-block only, without statin added as an ingredient.

4. Preoperative and stereotaxic surgical procedures

4.1. General surgical procedures

If the toxin was delivered to the MFB/SNc rats were injected with 25 mg/kg desipramine (i.p.) thirty minutes before and immediately after the operation to limit concurrent damage to noradrenergic pathways by 6-OHDA infusion (Roberts *et al.*, 1975). To prepare the surgical site, the scalp hair was clipped and the site disinfected with 70% ethanol. Body heat was conserved during surgery by placing the rat on a heating pad (Rex C10, Centre for Electronic Services, University of Stellenbosch). The rats were anaesthetized using a combination of ketamine hydrochloride (Anaket-V®, Bayer Healthcare, South Africa) and medetomidine hydrochloride (Domitor®, Pfizer, South Africa) (0.1 ml/100g, i.p.) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, USA). A small burr hole was drilled at the determined coordinates and a 22-gauge surgical-steel cannula was slowly inserted into the brain through which the toxin was infused using a 341-A model syringe pump (Sage Instruments, USA), running at an injection rate of 0.5 µl/min at the predetermined coordinates.

4.2. Establishing the coordinates for toxin injection into the medial forebrain bundle (MFB) of very young rats

The injection of the toxin (6-OHDA) entailed a scientific protocol to administer toxin to very young animals (weighing between 110–142g, see chapters 4). It was particularly important to perform initial experiments to establish a set of medial forebrain bundle (MFB) coordinates for the very young group of rats, since information on toxin injections into the MFB of young animals is very limited.

The stereotaxic coordinates were determined *a pirori* and verified in an animal of the same strain, sex and weight range, to ascertain the optimal location of the tip of the injection cannula (30G needle) in the MFB. A combination of ketamine hydrochloride (Anaket-V®, Bayer Healthcare, South Africa) and medetomidine hydrochloride (Domitor®, Pfizer, South Africa)

(0.1 ml/100g, i.p.) was used to anaesthetise the animals before placing them in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Following the Indian ink injection, the rat was decapitated and the brain placed in paraformaldehyde for 48h. The tissue was cut with a cryostat at a thickness of 30 μm. The sections were mounted onto clear glass slides and examined under a Nikon TE2000-E microscope (using a phase 4 resolution) to determine the location of the lesion, while referring to a stereotaxic atlas of the rat brain (Paxinos & Watson, 1986). The stereotaxic coordinates for the MFB injection delivered to the young animals were: 2.5mm posterior to Bregma, 1.8 mm lateral to the midline and 7.8 mm from the dura mater. These coordinates were used throughout the study for both toxin-induced and sham lesioned rats.

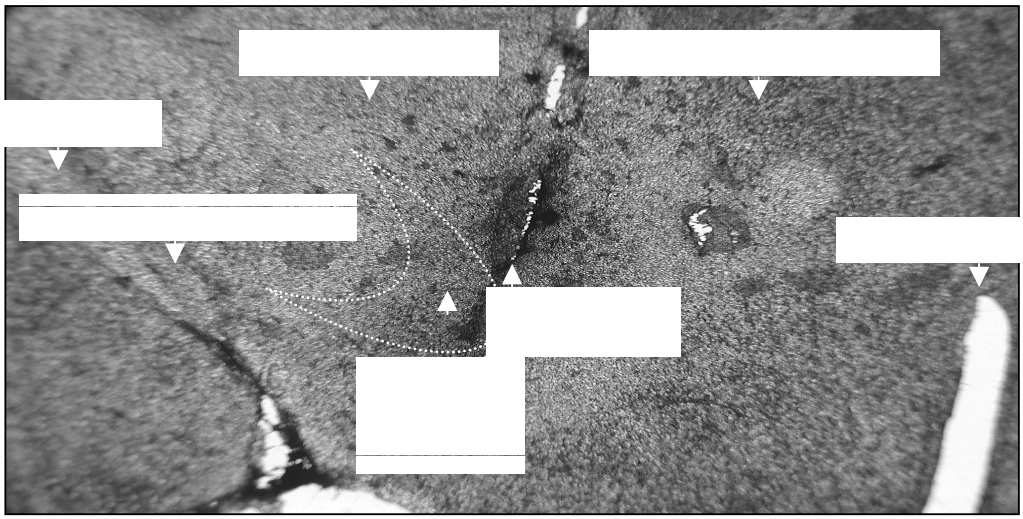

Fig. 1. The image shows the placement of the needle into the MFB, for the injection with 6-OHDA. The image was obtained with a Nikon TE2000-E microscope, the objective lens being × 4 with a Brightfield application attached, while the image was captured with a Sony 3CCD camera that was mounted upon the microscope.

4.3. Establishing the coordinates for the injection of the toxin into the striatum of very young rats

The brain stereotaxic atlas by Paxinos and Watson (1986) was referred to with no initial experiments performed to determine the coordinates for injecting the toxin into the striatum, since a large body of literature on this exists and the striatum is a relatively large structure. Bregma and Lambda were used to level the horizontal plane of the skull. From bregma, the following coordinates were used: anterior, 0.30 mm; lateral, 2.7 mm; ventral, 5.6 mm below the dura mater.

4.4. Establishing coordinates for toxin injection into the substantia nigra (SNc) of adult rats

For the surgical protocol employed in the study (as is described in chapter 5) the coordinates for inject through stereotaxic means were determined *a pirori* and verified in an animal of the same strain (Sprague Dawley, sex (male) and weight range (mature animals weighing between 300 and 320g at the beginning of the experiment). This was done in order to ascertain the optimal location of the tip of the injection cannula (30G needle) in the Substantia nigra compacta (SNc). The stereotaxic coordinates established for the SNpc injection were: 5.3 mm posterior to Bregma, 2 mm lateral to the midline and 7.5 mm from the dura. These coordinates were used throughout this particular study for all the experimental groups used.

4.5. The stereotaxic infusion of the neurotoxin and surgical post-care

Following the infusion of toxin, the needle was left in place for an additional 5 min, before slowly retracting it from the brain. The burr-hole drilled through the skull was filled up with sterilized oxidized cellulose (Ethicon, U.K.), the incision sutured and the wound coated with an iodine solution (Purdue Frederick, USA). The rats were placed in individual cages under heating-lamps until full recovery was achieved, after which they were returned to their home cages. The condition of the sutures, the rat's food and water intake and behaviour was monitored daily to ensure full recovery.

4.5.1. The infusion of 6-hydroxydopamine (6-OHDA)

6-OHDA solution (Sigma, St. Louis, MO) was kept on ice, and protected from exposure to light. The solution (the particular free-base weight of 6-OHDA, dissolved in 4 µl 0.1% L-ascorbic acid in 0.9% sterile saline) was infused over a period of 5 min using a 341-A model syringe pump (Sage Instruments, U.S.A.).

4.5.2. The infusion of hexahydro-2-isopropenyl-8,9-dimethoxychromeno[3,4-b]furo(2,3-h) chromen-6-one (Rotenone)

Rotenone is a major active ingredient and principal component of cubé resin that derives from *Lonchocarpus utilis* commonly used as a botanical insecticide and piscicide (Caboni *et al*., 2004). It is classified as a potent complex I (NADH:ubiquinone oxidoreductase) inhibitor that shows the ability to induce a PD-like syndrome in rats through the degeneration of the nigrostriatal dopaminergic pathway (Betarbet *et al*., 2000). Rotenone was freshly prepared, and protected from exposure to light. A stock solution of the rotenone solution was prepared by dissolving rotenone in the vehicle, that consisted of Dimethyl sulphoxide (DMSO):Polyethylene

glycol (PEG), with the vehicle that was prepared at a 1:1 ratio. Each rat received 3 µg of rotenone / 4 µl of vehicle. Rotenone is extremely hydrophobic, managing to cross biological membranes easily, and does not depend on a dopamine transporter for access to the cytoplasm of dopaminergic neurons (Betarbet *et al*., 2000; Greenamyre *et al*., 2001). It is also a well-characterized specific inhibitor of mitochondrial NADH dehydrogenase (Complex I), one of the five enzyme complexes of the inner mitochondrial membrane involved in oxidative phosphorylation (Betarbet *et al*., 2000; Thiffault *et al.*, 2000).

A combination of ketamine hydrochloride (Anaket-V®, Bayer Healthcare, South Africa) and medetomidine hydrochloride (Domitor®, Pfizer, South Africa) (0.1 ml/100g, i.p.) was used to anaesthetise the animals before placing them in a stereotaxtic frame (David Kopf Instruments, Tujunga, CA, USA). The surgical site was prepared by clipping the hair on the animal's head and disinfecting the site with 70% Alcohol (EtOH). The skin was sterilized using an iodophor (Betadine®, Purdue Pharmaceutical Products). A rubber heating-pad (REX C10, RKC, 19.0~99.9°C heat generator, Electronic Services, University of Stellenbosch) maintained the rat's body heat during surgery. Following the Indian ink injection, the rat was decapitated and the brain placed in paraformaldehyde for 48h. Forty-eight hours later the tissue was cut with a cryostat at a thickness of 30 µm. The sections were mounted onto clear glass slides and examined under a Nikon TE2000-E microscope (using a phase 4 resolution) to determine the location of the lesion, while referring to a stereotaxic atlas of the rat brain (Paxinos and Watson, 1986). The stereotaxic coordinates for the SNc injection established were: 5.3 mm posterior to Bregma, 2 mm lateral to the midline and 7.5 mm from the dura. These coordinates were used throughout the study for all experimental groups. Adult male experimental animals, weighing between 375-390 g were randomly assigned to their various experimental groups before surgery. A rat anesthetized with a combination of Ketamine and Domitor® was immobilized in a stereotaxic frame in a flat-skull position. A 2 cm midsagital skin incision was made through the scalp to expose the skull. An infusion cannula consisting of a sterilized length of 30 gauge stainless steel hypodermic tubing was stereotaxically placed via a hole in the skull and advanced so that the internalized tip was located within the SNc cell bodies. A stock solution of the rotenone solution was prepared by dissolving rotenone in the vehicle, consisting of Dimethyl sulphoxide (DMSO):Polyethylene glycol (PEG), with the vehicle that was prepared at a 1:1 ratio. Each rat received 3 µg of rotenone / 4 µl of vehicle. Rotenone is extremely hydrophobic, managing to cross biological membranes easily, and does not depend on a dopamine transporter for access to the cytoplasm of dopaminergic neurons (Betarbet *et al*., 2000; Greenamyre *et al*., 2001). It is also a well-characterized specific inhibitor of mitochondrial NADH dehydrogenase (complex I), one of the five enzyme complexes of the inner mitochondrial membrane involved in oxidative phosphorylation (Betarbet *et al*., 2000; Thiffault

et al., 2000). The solution was infused through a tube attached to a Hamilton syringe over a period of 5 min using a 341-A model syringe pump (Sage Instruments, USA). Following the infusion, the needle was left in situ for an additional 5 min, before slowly retracting it from the brain. The drilled burr-hole through the skull was filled with sterilized oxidized cellulose (Ethicon, U.K.), while the incision was sutured and again coated with iodophor. To recover, the rats were placed in individual cages under heating lamps and a post-operative pain reliever (Temgesic 0.01 mg/kg, i.p.) was administered. The condition of the sutures, the rat's food and water in-take and behaviour were monitored daily to ensure full recovery.

5. Specific tests used for assessing rodent behavior

The following tests were used to elicit certain behaviors that have proved to be a reliable and valid indication of the degree of DA loss sustained from the lesion. A significant proportion of behavioral testing was carried out drug-free, since it reflects a more natural response following the lesion (Roghani *et al*., 2002).

5.1. Forelimb use asymmetry ('cylinder') test

Limb use asymmetry during vertical-lateral exploration of the walls of a cylindrical chamber correlates to the extent of nigrostriatal terminal loss (Schallert & Woodlee, 2005; Schallert, 2000). Rats were placed individually in an upright transparent Plexiglas cylinder that had been placed on a tabletop. The diameters of the container measured at 19 cm across and 24.4 cm in height. The walls were high enough to prevent the animal from reaching the top edge when rearing by standing on its hind limbs and were wide enough to permit a comfortable 2 cm space between the tip of its snout and the base of its tail when all four paws were placed on the ground. The floor of the container consisted of a transparent sheet portraying four equal quarter-circular sections. The animals were tested during the light cycle (between 10h00 and 12h00) with each testing session lasting 5 min in total. This also served to prevent the rats from habituating to the cylinder environment, lest they should become passive (Schallert *et al*., 2000).

Following the assessment, each rat was immediately returned to its home cage. A video recorder with slow motion and frame-by-frame capabilities was used to analyze and rate the rat's behavior in the cylinder at a later stage. The camera was mounted on a tripod at an angle and underneath the cylinder, to allow a full-view of all the animal's activities, even when turning away from the camera as well as its movements on the floor of the cylinder. This test was also referred to as 'the small open field maze' (chapter 6).

A number of parameters were included for quantifying the degree of forelimb-use asymmetry shown. Each of these will now briefly be discussed.

5.2.1. The independent use of the forelimbs

The independent use made of the forelimb is defined as the active use made of a single forelimb, while the other remains stationary but in constant contact with the surface of the cylinder wall. The rater was blind as to the condition of the animals and scored limb use as number of times that the left (ipsilesional), right (contralesional), or "both" limbs were placed on the wall surface or moved laterally during vertical exploration. The score of each was expressed relative to the total amount of times that the forelimbs (ipsilesional + contralesional + both) was placed and by multiplying this with 100 to present the values as percentages (Connor *et al*., 1999). In cases where it was unclear whether the limb had been placed independently or simultaneously, it was neglected for evaluative purposes.

5.1.2. Independent forelimb exploration ('wall stepping')

Forelimb use during explorative activity was analyzed by videotaping rats in a transparent container of a cylindrical shape, of diameter19 cm across and 24.4 cm in height. The container neither had a top nor a bottom. 'Wall stepping' was defined as the rat making independent use of either the right or the left forelimb for making initial contact with the wall, followed by subsequent 'stepping' movements in a lateral direction, when the animal moved in a vertical posture by shifting its weight side-ways by using its hind limbs. The number of wall stepping movements performed with the rat's contralateral (to the lesioned hemisphere) forelimb was compared to the number of times this was performed by the ispsilateral (to the lesioned hemisphere) forelimb.

5.1.3. The amount of steps taken by the hindlimbs

The amount of steps taken by each of the hindlimbs (ispsilateral vs. contralateral), in either a forward or a backward direction were counted.

5.1.4. The defecation-urinary (DU) index

A defecation and urination index was determined based on the total sum of defecation boli and urination spots observed.

5.1.5. The frequency of vertical rearing

The number of rearing (standing on hind limbs without touching the wall of the cylinder (19 cm in diameter) was also recorded. The height of the cylinder was such that it prevented the animal from reaching the top edge when rearing on its hind legs. The amount of rearing displayed by each animal was scored. This was counted each time the rat reached a full-rearing posture, with neither of the front-paws making contact with the inside of the wall. Together with the DU index,

vertical rearing serves as robust measures of the emotional status of the animals (Gray & Cooney, 1982; Pijlman *et al*., 2002).

5.1.6. The number of non-drug induced (spontaneous) half-turn rotations

The floor of the cylinder was divided into 4 equal-sized quarters. The amount of times that the rat rotated towards the side of the lesion (ispsilateral) as well as away from the lesion (contralateral) were counted, with a rotation consisting of a continuous 180° turn.

5.2. The vibrissae-evoked forelimb placing test

There are various experimental advantages that the vibrissae sensory system offers for analysis of motor control. Whisker motor-neurons are located in the lateral facial nucleus with numerous structures providing input to the lateral facial nucleus, the most mysterious and important one being the putative central pattern generator (CPG). Although recent studies identified candidate structures for the CPG, the precise identity and the functional organization of this structure remains uncertain. The vibrissae cortex (VMC) is the largest motor representation in the rodent brain, and recent work has clarified its localization, subdivisions, cytoarchitectonics and connectivity.

This test evaluates the degree of sensorimotor integration that occurs across the two hemispheres. Intact rats instinctively attempt to place their forelimb(s) upon any nearby surface when their whiskers are stimulated (Woodlee *et al*., 2005). Consistent with parkinsonian akinesia, unilaterally 6-OHDA-lesioned rats fail to evoke a placement response from the limbs regardless of the sensory trigger, while the contralateral vibrissae maintain the ability to reliably trigger limb placement.

In this test, the experimenter held the rat by the torso, thereby allowing the forepaw being tested to hang freely while the untested forelimb was gently restrained. Each forelimb was tested independently by orienting one side of the animal towards a tabletop and moving the animal in a slow lateral direction toward the edge until the vibrissae of that side made contact with the table's edge (Schallert & Tillerson, 1999; Barth *et al*., 1990). In an intact animal the stimulated vibrissae will cause the rat to instinctively place the corresponding forelimb onto the tabletop, while, with lesioned animals, placing may not occur at all (scoring '0'), indicating a failed motor response towards a spatio-sensory stimulus, or there is a delay between stimulation and placing. Ten trials of each forelimb were performed during the testing session. An experimenter, blind to the experimental condition of the animals, recorded the number of successful placing reactions by each forelimb.

5.3. The bracing (‘adjusting steps’) test

The rats were monitored for their ability to make postural adjustments in response to an imposed weight-shift by using an isolated forelimb test (initially described by Schallert *et al.*, 1979; 1992). The test is useful for monitoring lesion- and transplant-induced changes in forelimb motor function, which is a behavioral parameter that may be analogous to limb akinesia and gait problems seen in patients with Parkinson's disease (Olsson *et al.*, 1995). Briefly, the experimenter suspended the rat above a tabletop surface, with the hind limbs and one forelimb restrained in one hand. By doing so, the rat supported most of the weight of its body with the unrestrained forelimb. While the experimenter slowly moved the animal at a constant rate (300 cm in 30 sec), the number of adjusting steps taken by each forelimb was recorded by the experimenter, who was blind to the treatment of the rat. This procedure was performed across two conditions, namely when the animal was moved along the table-surface in the lateral forward direction (leftward movement), as well as in the lateral backward direction (rightward movement). Two trials for each animal were performed, with each trial consisting of the rat being moved once in each direction. The mean score of the two trials was considered a representative result for each animal.

5.4. The isolated forelimb (‘catch-up’) stepping test

The test provides a useful means for evaluating an animal’s ability to make postural adjustments in response to weight shifts imposed by the experimenter handling the rat. The test was initially described by Schallert *et al.*, 1979; 1992; Olsson *et al*., 1995 as a way with which to monitor lesion- and transplant-induced changes in forelimb motor function, and is seen as a behavioral parameter that may be analogous to postural instability or akinesia seen in patients with PD (Olsson *et al.*, 1995). The experimenter held one forelimb and both hind-limbs off the surface of a tabletop, while permitting one forelimb to hold the animal’s full weight. The animal was then moved laterally (held in the position described) first in one direction (leftward), and then in the opposing (rightward) direction. The number of catch-up (adjusting) steps taken by each of the forelimbs when slowly moved at a constant rate (300 cm in 30 seconds) was counted. A trial consisted of moving the rat twice in both directions.

5.5. The standard open field maze

Although the Open Field maze classically functions as a paradigm used for measuring levels of anxiety in animals, its can also be used for measuring the degree of locomotor activity displayed by animals (Prut & Belzung, 2003). The behaviour of each rat was recorded for 5 minutes. As

was previously described by Ramos *et al.* (2002), each rat was individually tested and to signal the start of the test, the rat was placed on the outer-left corner of the arena with the snout pointing towards the inner zone. The activities of the rat were video-recorded and subsequently analyzed with Noldus EthoVision software (Noldus Information Technology, V 3.0). The frequency of rearing that the animals exhibited, the total distance traveled (cm), the mean velocity (cm/s) of their movement and the total duration (in seconds) that the rat spent moving, were regarded as constituting an index of locomotor activity.

5.6. The apomorphine-induced rotation test

The degree of apomorphine-induced stereotypic rotations were assessed. All rats received three tests of apomorphine-induced rotation: on the day prior to starting statin/placebo therapy (lasting for 14 days), the day before surgery, and on the 5th day post-infusion. Animals with severe unilateral dopamine depletion exhibit asymmetrical rotational behavior in a contraversive direction in response to the dopamine agonist apomorphine (Borlongan *et al*., 1995; Ungerstedt and Arbuthnott, 1970). Rats were tested for rotation-asymmetry by challenging with 0.75 mg/kg apomorphine hydrochloride (Sigma), dissolved in 0.2% ascorbate in 0.9% saline, injected subcutaneously (in the neck). Thirty min after injection the animal was placed in a spherical rotometer (diameter 25 cm) for 60 min. The animals were video-recorded from a ventral perspective, and their behavior analysed with a macro designed in the Noldus Ethovision software (Noldus Information Technology, The Netherlands). For each assessment, the total number of 180° turns in left and right directions was counted during the 60 min interval that the trial lasted. Analysis of data was based on net ipsilateral (right-left) turns and on net contralateral (left-right) turns. The amount of clockwise half-circle (180°) rotations (ispsilateral to the lesion) was scored as positive turns, and counterclockwise turns were counted as negative turns. The net rotational asymmetry score was calculated as the difference between clockwise and counterclockwise turns over the 60-min duration of the trial following the injection of apomorphine (Sun *et al*., 2005).

6. Animal sacrifice

At the pre-determined terminal endpoint of each experiment, the rats were killed by a method deemed appropriate for the particular neurochemical analysis that was due to be performed on the collected brain tissue. All treatments given confirmed to international ethical standards and the protocol has been approved by the local committee for Experiment Animal Research of the University of Stellenbosch and are were accordance to the National Institutes of Health Guide for Care and Use of Laboratory Animals.

6.1. Decapitation

Upon reaching the experiment's terminal end-point, certain animals were decapitated and the brain tissue removed, since this particular method for terminating the life of the animal was the most rapid and efficient, and also represented the most appropriate for the particular biochemical analyses to be performed on the collected brain tissue.

An hour preceding decapitation the animals were moved from their home cage to another cage and transferred to a room isolated from all the other rodents. No anaesthesia was given prior to this method of killing since it would modify the biochemical activity of the brain tissue. Any anaesthetic agents will contaminate the samples and obscure the effects of the experimental manipulations that had been given (Carney & Walker, 1973; Barna *et al*., 1993; Carlberg *et a*l., 1995; Illera *et al*., 2000).

The guillotine was placed upon a clean and stable benchtop. Each rat was brought into the dissection room individually so the rest of the colony could not detect the blood odor. The rat was gently restrained, but with every effort made to adjust the transport of the rodent until it appeared calm. The rodent was held securely, and placed on the stage at the entrance to the guillotine. The animal was gently, but assertively pushed forward until its head was securely in the guillotine apparatus. As soon as the head was in position, it was verified that the the head is completely through the opening of the guillotine. The guillotine lever was smoothly and quickly depressed, thereby decapitating the rodent. The rest of the carcass was disposed of in accordance with the regulations of the local animal ethics committee (University of Stellenbosch).

6.2. Transcardial perfusion

The effect of the injections on dopaminergic neuron survival was evaluated in rats belonging to each group (experimental versus control) using transcardial perfusion, a method commonly used for fixating tissue in immunohistochemical localization protocols. The method takes advantage of the subject's circulatory system to deliver the fixative solution evenly throughout the body tissues, with optimal penetration of the brain. The method is particularly suited for evaluating tyrosine hydroxylase immunoreactivity (TH-ir, and discussed below) since the fixation preserves neuronal ultrastructure, and stabilizes protein and peptide conformation so that antibodies can bind to antigen sites.

6.2.1. Perfusion solutions

6.2.1.1. Pre-fixation solution: Phosphate buffer stock solution (PBS)

The ×10 stock solution was made by dissolving 80 grams NaCl, 2 grams KCl, 14.4 grams of Na_2HPO_4 and 2.4 grams in 800ml of distilled water. The pH was adjusted to 7.4 with 5M NaOH. Distilled water was added to make up the solution to 1 litre. A 0.15M PBS working solution was prepared by mixing the stock solution with distilled water to a ration of 1:10.

6.2.1.2. Fixation solution: 4% paraformaldehyde solution in 0.15M PBS

40 grams of paraformaldehyde were mixed in 800 ml of 0.15M PBS, which had been made from the stock solution. The mixture was covered and mixed on a stirring magnetic plate with the heat set at 40°C and the mixture continuously stirred until the solution was completely clear. The solution was prepared under a fume hood.

6.2.1.3. Post-fixative solution: 20% sucrose in 0.15M PBS

200 grams of sucrose was dissolved in 800 ml of 0.15M PBS working solution made from the stock solution described earlier. The solution was made up to 1 litre.

6.2.2. Perfusion procedure

The perfusion pump was set up and the perfusion needle (catheter) was attached with the valve kept closed. A 100 ml of distilled water was run through the system (consisting of the bottle and tubing) with the aim to remove any residue that may have remained from previous use. The pump bottle was filled with 200 ml of pre-fixation solution (PBS) of room temperature (21-23°C). The valve of the tubing was briefly opened, allowing the solution to drip slowly and steadily (20 ml/min), and the valve closed then again. The surgery site was set up, complete with tools, guillotine, specimen bottle (which contained the 30% sucrose solution). A rack (the top cage of a standard rat holding box) was placed over the sink and the tap opened to allow water to drip at a steady pace.

Rats were deeply anaesthetized by administering 30% urethane anesthetic (1 cc/100 g, IP), and the pinch-response method was used to determine the depth of anesthesia. Great care was taken to ensure that the animal was completely unresponsive before proceeding with the procedure. The animal was placed on the rack, and an incision was made with sharp surgical scissors through abdomen comprising the length of the diaphragm. Using the same pair of scissors, a cut was made through the connective tissue at the bottom of diaphragm to allow access to rib cage. With the blunt side of the scissors facing down, the ribs were cut through just left of the rib cage midline. A single center cut was made through the rib cage to open up the thoracic cavity. The rib cage was secured with mosquito clamps to expose the heart and provide drainage for blood and fluids.

With the heart still contracting and diluting, the heart was held steady and a catheter needle

inserted directly into the protrusion of the left ventricle and extend straight up ≈ 5 mm. The needle position was secured by clamping it in place. The catheter valve was allowed to slowly release the fluid, delivering the pre-fixative solution (PBS) at a steady flow rate of ≈ 20 ml/min. A cut was made through the atrium with sharp scissors, and care taken to make sure the solution was flowing freely.

As soon as exsanguinations had been achieved (when it was seen that blood had been cleared from the animal's body), infusion with 10% paraformaldehyde (200 ml) commenced. Spontaneous movement (the so-called 'formalin dance') and the lightened color of the liver were taken as good indicators of clearing of blood from the body, and replacement with formalin.

6.3. Brain tissue collection

The animal was then removed from the rack and decapitated as close as possible to the back of the ears. A cut was made with sharp scissors from the base of the head up to the level of the eyes. The skin was pulled away from the skull and the skull plates removed with tweezers to expose brain. Any remaining dura was cut away with small sharp scissors. The flat end of a spatula was carefully slid underneath the brain, and the optic, olfactory, and cranial nerves severed. The brains were rapidly removed from the skull with the spatula and placed in a specimen bottle containing the 30% sucrose solution that offered cryoprotection. The brains were stored at 4ºC in the 30% sucrose solution for ≈ 48 hrs until they had descended to the bottom of the container, indicating complete tissue penetration with the solution, they were removed, the brain tissue blocked, placed in plastic covers, frozen over liquid nitrogen vapor and kept at -80°C until sectioning.

7. Brain dissections

The brains were rapidly removed from the skull by means of the flat end of a spatula and lifted out of the brain case. The respective areas were dissected out as follows:

7.1. The striatum

The total left and right striatum was dissected out on an ice-cooled dissection slab (cooled by liquid N_2 fumes) by cutting coronally at the level of the infundibular stem, thereby forming a forebrain block containing the striatum. The forebrain was bisected along the midline and cortical tissue for each hemisphere peeled back to expose the striatum, which was dissected along the boundary of the corpus callosum.

7.2. Substantia Nigra pars compacta (SNpc)

The dopaminergic neurons located in the SNpc act as important regulators of corticostriatal neurotransmission. In the neuropathology seen in PD, progressive loss of these neurons leads to debilitating motor dysfunction (Chichung Lie *et al*., 2002). The SN was dissected using a dissection microscope, taking care to avoid contamination by ependymal and subependymal cells by complete removing the tissue adjacent to the 3rd ventricle. The two half sections (collected from both cephalic hemispheric halves) were used as a matched pair of slices: one half having had received the injection with the toxin/vehicle, and the other half that was used as an internal control.

8. Investigations to determine the neurochemical content

8.1. High Performance Liquid Chromatographic (HPLC) analysis for analysing Dopamine (DA) and DA Metabolites (DOPAC & HVA)

After the respective lesions were complete, at two weeks following a lesion made to the MFB, and four weeks following lesioning of the striatum, all animals were killed by decapitation. The brains were rapidly removed from the skull and the total striatum was dissected out on an ice-cooled dissection slab. This was done by cutting coronally at the level of the infundibular stem, thus forming a forebrain block containing the striatum. The forebrain was bisected along the midline and cortical tissue for each hemisphere peeled back to expose the striatum, which was dissected along the boundary of the corpus callosum. The striata were snapped frozen in liquid nitrogen (-198 ºC) until analysis for DA and its metabolites by using HPLC-EC.

Dopamine (DA),and DA-metabolites (3, 4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), contents of the left and right striatum were determined by the same HPLC-EC method that has previously been described by Harvey *et al.* (2006). The chromatographic system consisted of a GBC LC 1120 HPLC Isocratic pump that contained an inlet filter and a Rheodyne 7725i injection valve comprising of a 50 µl loop for introducing samples to the system. For separation a 5 µm C_{18} Phenomenex Luna reverse phase analytical column (150 × 4.6 mm) was connected to a GBC LC 1260 Electrochemical Detector (+0.6 V) as well as a Spectra-Physics SP4290 integrator. The mobile phase consisted of 0.1 M sodium formate buffer, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM sodium heptane sulphonic acid, 6% v/v methanol and 4% v/v acetonitrile. Milli-Q water was used to make the solution up to 1000 ml and the pH set at 3 by adding 6 ml of orthophosphoric acid (85%). The composition was degassed twice prior to use. The flow rate of the mobile phase running through the system was set at 1.2 ml/min. The temperature remained ambient throughout. On the day that analysis took place the samples were allowed to thaw and the weight of each sample was recorded. A total volume of 1 ml 0.1 M perchloric acid solution was added to each tube, sonicated and then placed on ice for 20 min, whereafter the tissue was centrifuged (20 min, 4˚C,

24 000 × g, Sorval Discovery 9056 ultracentrifuge). Following the withdrawal of 200 µl aliquot of supernatant, 20 µl of the internal standard solution (isoprenaline HCL) was added and the solution was mixed. A sample of 50 µl was injected into the column for direct content analysis. Standard dopamine and metabolites (DOPAC and Homovanillic Acid or HVA) solutions were prepared in a vehicle of 0.5 mM sodium metabisulphite and 0.3 mM Na_2EDTA diluted with perchloric acid (0.1 M). Monoamine standards ranged from 10 ng/ml – 200 ng/ml and linear standard curves were found in this particular range. The monoamines and metabolites were quantified by comparing the area under the curve (AUC) of the sample/ AUC of the internal standard (isoprenaline) to the monoamine standard, utilizing the linear equation obtained from the standard curve. Chemstation Rev A 08.03 software was used for data acquisition and analysis purposes. The monoamine concentrations were expressed as ng/g wet weight (ww) of tissue (mean ± S.E.M.).

8.2. Tyrosine hydroxylase immunohistochemistry (TH-ir)

To stain for tyrosine hydroxylase, free-floating coronal sections of 60µm thicknesses were cut with a cryostat at −20 °C. The sections were quenched by incubating with 0.3% hydrogen peroxide for 30 min at room temperature, in a humidifying container. This was followed by a succession of washes (5 ×10 min) with PBS (0.15 M, pH 7.4). The sections were placed in a 10 mM citrate buffer (pH 6.0) and placed in a microwave oven for 15 min, at full-power. The sections were then left to cool, before the wash-cycle was repeated. The sections were then pretreated with 150 µl horse blocking serum, and 0.1% Triton X-100 for 30 min, before application of the primary monoclonal anti-TH (BrdU mouse monoclonal (dilution 1:500, DiaSorin, USA) and overnight incubation at 4ºC. The next day the slices were washed in PBS (5 ×10 min), before applying the secondary biotinylated antibody (dilution 1:400) for 1 hour at room temperature. The wash routine was repeated, before incubating the slices with peroxidase labeled Mouse IgG ABC reagent (ABC kit, DakoCytomation, USA, with concentrated diaminobenzidine (DAB) as the chromogen). Tissue sections were incubated at room temperature until staining developed. The sections were given a final 15 min rinse with distilled water, before dehydrating them with 96-100% alcohol (EtOH), clearing with Xylol (Merck, South Africa) and mounting the sections onto clear glass slides.

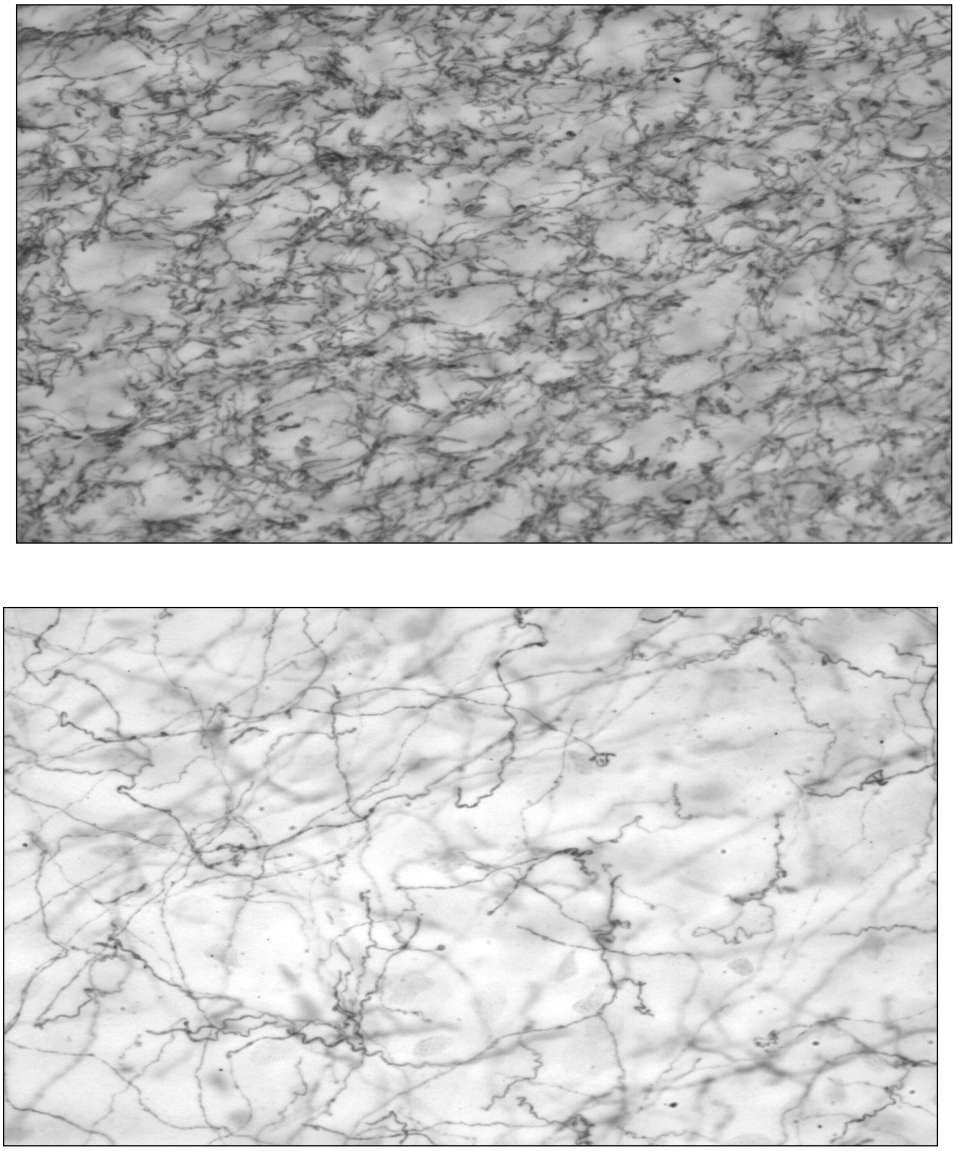

Fig. 2.1. The figures portray examples of photographs obtained with a Nikon TE2000-E microscope, the objective lens being × 4 with a Brightfield application attached. The photographic image was captured with a Sony 3CCD camera that was mounted on top of the microscope. Figure A shows the typical image of an intact hemisphere, with dense populations of DA-ergic neurons remaining, following either no infusion or having merely been infused with vehicle (i.e. saline or DMSO:PEG). Figure B shows the consequences of exposure of DA-ergic neurons to neurotoxins (i.e. 6-OHDA or rotenone). As can be seen, when comparing this image to that of figure A, the DA-ergic neurons that had survived the toxin infusion make up for a very sparse population.

9. Proteome contents analysis

9.1. Proteomics subfractionation: Isolation of the mitochondria

The tissue samples, stored in Eppendorf containers, were removed from the -80°C fridge and placed on ice. Mitochondrial fractions were isolated from dissected brain tissue using a

commercially available kit (Sigma-Aldrich, USA). The tissue was weighed and then cut into smaller pieces with a scalpel-blade on a glass plate cooled with liquid Nitrogen vapours. The samples were pooled and homogenized with a glass homogenizer in 10 volumes of extraction buffer. The buffer contained 10mM HEPES, 200mM Mannitol, 70mM Sucrose, 1mM EGTA and Bovine Serum Albumin for removing excess lipids, with the pH set at 7.5. The homogenate was transferred to a 2ml Eppendorf tube and kept on ice. In order to obtain a mitochondrial fraction of ultra-high concentration, and to minimise the presence of contaminants, such as lysosomes or peroxisomes, the homogenate was centrifuged at a differential gradient. Centrifugation first occurred at a low rate of 1000 × *g* for 5 min, after which the supernatant liquid was transferred to a new tube and then centrifuged again at a higher rate of 3500 × *g* lasting for 10 min. The supernatant was removed and the pellet resuspended in 10 volumes of extraction buffer. The centrifugation steps were repeated, and the pellet resuspended in storage buffer, containing 10mM HEPES, 250 mM sucrose, 1mM ATP, 0.08 mM ADP, 5mM sodium succinate, 2mM K_2HPO_4, and 1 mM DTT, pH 7.4.

9.2. Protein extraction and purification

The supernatant was purified using a ReadyPrep Cleanup Kit (Bio-Rad Laboratories, USA), and the protein concentration of the sample quantified with a Model 550 Microplate reader and RC DC Protein Assay Kit (Bio-Rad Laboratories) using BSA as the standard. Aliquots of the final protein solution were stored at -80˚C until further use. Two-dimensional gel electrophoresis (2-DE) is a protein separation technique that allows for the separation of proteins according to charge (Isoelectric focusing, IEF) in the first dimension and by molecular mass (SDS-PAGE) in the second (Mary, 1999). 2-DE was performed on mitochondrial proteins derived from the SN in order to assess differential protein expression between the placebo + vehicle-infused and the statin-treated + vehicle-infused groups of animals.

9.3. Electrophoretic protein separation

The extracted mitochondrial proteins were separated by IEF followed by 2-D SDS-PAGE. The first dimension separation was performed by mixing the appropriate amount of protein sample with a rehydration buffer, containing 8 M urea, 4% (w/v) CHAPS, 65 mM DTT, 0.2% (v/v) Bio-Lytes and a trace of bromophenol blue. The sample was loaded in the IEF focusing tray, followed by the IPG gel strip gently being placed gel side down onto the rehydration solution and overlaying the IPG strip with 3 ml of mineral oil to prevent evaporation taking place during the rehydration process. The focusing tray was placed on a PROTEAN IEF Cel (Bio-Rad Laboratories, USA) and the software program was then run. Following IEF, the strip was

equilibrated for 15 min in the equilibration buffer containing DTT also, followed by an additional 15 min in equilibration buffer II containing iodo-acetamide.

9.4. Gel staining for protein expression

For the second dimension separation, the gel strip was placed on the upper edge of the 4-12% separation gel with its backside pasted tightly along the surface of the glass plate. 5 µl of low molecular weight protein marker was added at one end of the IPG strip. Low-Melt Agarose was used for fixing the gel strip and to remove air bubbles. The gel was run at 200V, until the bromophenol blue ran clear of the gel strip and condensed in a single line. As soon as the bromophenol blue front had traversed the gel, electrophoresis was stopped.

9.5. Image acquisition and data analysis

The strip size was 11 cm and had an ampholyte range of 5-8 (Bio-Rad Laboratories, USA). Completed polyacrylimide 2-D gels were stained with G-Colloidal concentrated Coomassie Brilliant Blue-R (Sigma-Aldrich, USA) to visualize the separated proteins. Gels were scanned using the GS-800 Calibrated scanner (Bio-Rad, USA) and the Quantity One - 4:5.2 (Basic) software program (Bio-Rad, USA) employed to create gel images. These gel images were subsequently analysed using the PDQuest (version 8) software program (Bio-Rad, USA) to identify the differentially expressed ($p< 0.05$, student's t-test) spots between control and statin-treated animals.

9.6. Destaining, in-gel digestion and peptide extraction

For identifying the protein profile, the spots of interest were subjected to Mass Spectrometric (MS) analysis. Briefly, the gel pieces containing the proteins of interest were manually excised from the 2-D gels followed by subjection to in-gel enzymatic digestion using trypsin (Shevshenko *et al*., 1996). Using the manufacturer's protocol, the gel plugs were processed by a MassPrep Robotic Protein Handling System (MS Technologies, UK). The samples were placed in a 96-well polypropylene microtiter plate and destained twice, using 50 % acetonitrile in 50 mM ammonium bicarbonate, followed by a rinse with acetonitrile and allowed to air dry for 10 min. They were then reduced with 10 mM dithiothreitol for 30 min, followed by subjecting them to alkylation with 55 mM iodoacetamide. These steps were followed by rinsing the samples three times with acetonitrile, followed by 100 mM ammonium bicarbonate, and finally by acetonitrile. Digestion was performed by adding to each sample a 25 µl aliquot containing 6 $ngµL^{-1}$ trypsin. The samples were then allowed to incubate for 4.5 h at 37 °C. The initial extraction of the peptides were done using 30 µL of an aqueous solution containing 2%

acetonitrile and 1% formic acid. A second extraction using 15 µL of an aqueous solution containing 51% acetonitrile and 0.5% formic acid was then performed and combined with the first extraction in a cooled second 96-well plate and stored at –80^{o}C prior to analysis by mass spectrometry.

9.7. Peptide separation by means of in-line Liquid Chromatography and Electrospray Ionisation Mass Spectrometry (EI-MassSpect)

The partially digested peptide mixture was contained in a 96-well microtitre plate, which was transferred to a modular CapLC and autosampler system (MS Technologies, UK). Using the method described by Wilm *et al.* (1996) and Chen *et al*. (2006), the eluted peptides were then analysed further using quadripole time of flight (ESI-QUAD-TOF) mass spectrometry (MS Technologies, UK) fitted with nano-electrosprayer having an applied capillary voltage of 3.5 kV. Prior to analysis, the instrument was calibrated against a collisionally induced decomposition (CID) spectrum of the doubly charged precursor ion of [glu^{1}]-fibrinopeptide B (GFP). Calibration was accepted when the error obtained on all subsequent acquisitions was < 20 ppm, while the sensitivity of the machine was assessed by detecting a 500 fmol injection of GFP, with a base peak signal:noise ratio of > 50:1 on the doubly charged ion. Throughout analysis, the sensitivity and calibration of the MS instrument as the chromatographic resolution of the GFP peak were checked at regular intervals. For recording spectra, the MS instrument was operated in data dependent acquisition (DDA) mode over the mass/charge (m/z) range of 50-2000. During the DDA analysis, both MS and tandem mass spectrometry (CID) was performed on the most intense peptides as they eluted from the column.

9.8. Homology protein searches using bio-informatics tools

Following data acquisition, all the individual uninterpreted MS/MS spectra that had been acquired for each of the precursors within a single LC run were combined, smoothed, the background subtracted, centred, deisotoped and centroided automatically using the ProteinLynx Global Server search engine (MS Technologies, UK). The MASCOT^{TM} peptide identification search algorithm (Perkins *et al*., 1999) was used for querying the nonredundant database at the National Center for Biotechnology Information (NCBInr) with the ProteinLynx Global Server output that was generated as a single Mascot-searchable peak list file. The search parameters were defined to include up to two missed cleavage sites, a 100 ppm tolerance against the database-generated theoretical peptide and product ion masses, and a minimum of a single matched peptide. A list of the twenty highest scoring entries was produced and each suggested

protein identification was either confirmed or rejected by comparing the theoretical sequence with the observed MS/MS data. The generated dataset was searched against Matrix software (http://www.matrixscience.com/cgi/), by using a rat reference database, the IPI rat version 3.34 (http://www.ebi.ac.uk/IPI/IPIrat.html, released 02/10/2007). Furthermore, fragments were sequence-identified by performing a database search against several publicly available databases, i.e. SwissProt, www.ebi.ac.uk/swissprot; NCBI nr, www.ncbi.nlm.nih.gov; and ENSEMBL (http://www.ensembl.org/index.html). BLAST searches (www.ncbi.nlm.nih.gov/BLAST; http://www.arabidopsis.org/Blast/) were performed on the peptide fragments obtained earlier, to further analyze non-annotated expressed sequence tag (EST) hits (Altschul *et al*., 1990).

10. Drug treatment regime

For the experiment described in chapter 7, a drug treatment protocol was followed prior to the unilateral infusion of rotenone into the SNc. The drug was administered orally on a daily level for 2 week, with the drug administered at a fixed time every day (between 13:00 and 13:30). Prior to starting the drug regime, two weeks was allowed for training the rats to eat a jellie-cube that consisted of flavoured sugar-water dissolved in hot water and gelatine (the gelatine was used for setting the solution rapidly). A drug-treatment regime was then followed that consisted of dissolving 6 mg of the active chemical component of simvastatin in 50 µl of absolute Ethanol (EtOH) in an Eppendorf microtube, which was mixed with the jelly-solution. For preparing a single jellie-cube, a droplet of the solution was placed with a Pasteur pipette in an ice-maker holder, while 50 µl of the EtOH-dissolved statin-solution was re-suspended in the sugar solution with a sterilized pipette. After the solution had set completely, the jelly-cube was removed from its plastic holder, and a single cube was given to each rat. Control rats were treated with placebos, consisting only of a jelly-block, however with an equal amount of ethanol as the statin-treated rats had received.